The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 53 APRIL, 1959 NO. 4

The Official Publication of the Central Society for Clinical Research

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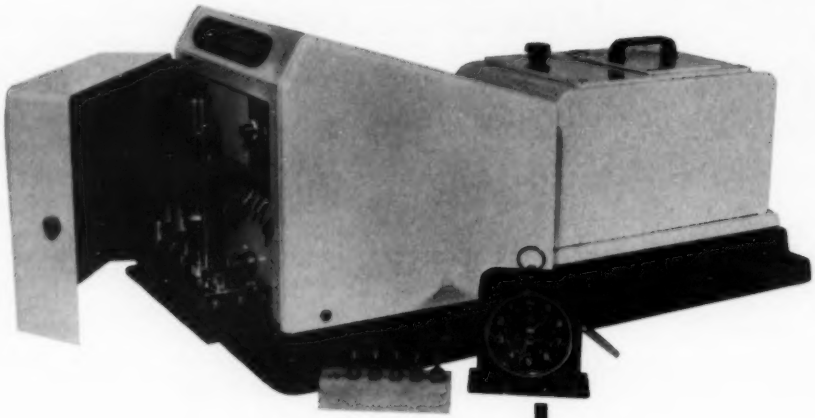

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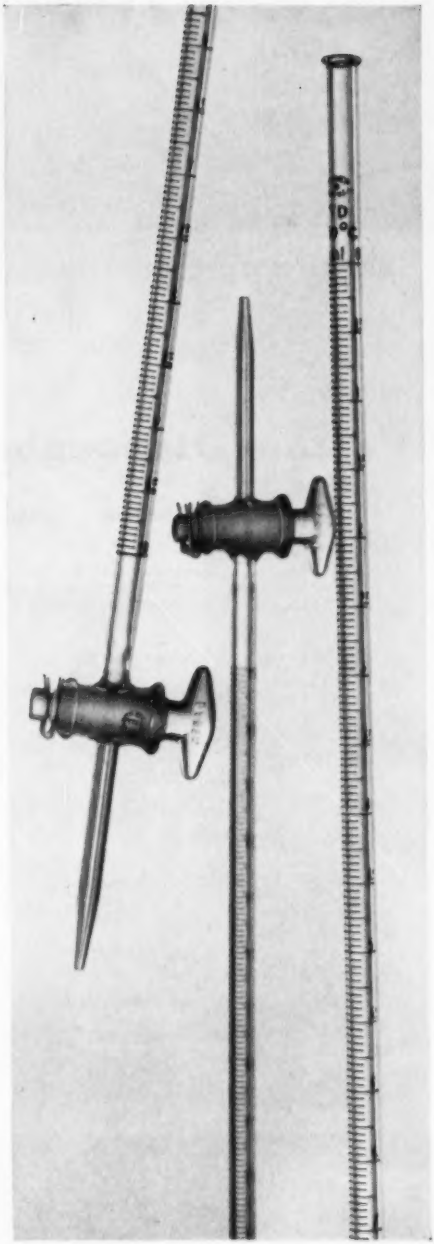

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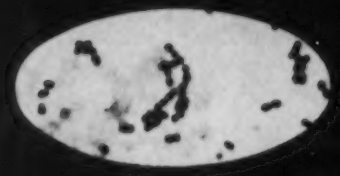

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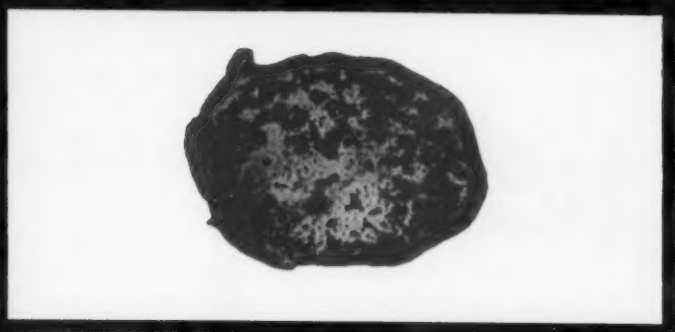

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Aside from the particular product or service offered by a manufacturer, long-term success is, after all, dependent on the guarantee of reliability and quality offered the consumer. Knowing this, industry has developed rigid quality control over production. Extensive testing, taking weeks or months, is undertaken before release of a product run. This testing serves a dual purpose. First, efficiency in manufacturing is maintained and guarded; secondly, the product or service offered is kept at a standard to satisfy the discriminating public.

In the clinical laboratory, advances made in specific instrumentation, methods, technique, and diagnostic reagents have, until recent years, greatly exceeded the overall quality control practiced over biochemical determinations.

It is not surprising that modern advances and antiquated control are functioning side by side in laboratory testing. *Adequate control measures have not been available.*

Now, two major steps have been taken to fill this void. First, the application of statistical measures of precision and of accuracy, and the routine use of the specific deviation yardstick are becoming widespread *in revealing* laboratory efficiency problems. Secondly, the common usage of two important standards-in-serum for biochemical testing, Versatol (normal) and Versatol-A (abnormal), with known absolute values in the normal and abnormal range, meet the demands of advanced laboratory analyses.

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Earlier, in this same Symposium, pioneers in microchemistry recommended Versatol and Versatol-A for the *standardization* of the newest microchemical methods.

Quality Control in Clinical Chemistry was a featured presentation earlier this month at the American Association of Clinical Pathologists meeting in Chicago. The use of freeze-dried reagents *like* Versatol and Versatol-A was recommended for routine daily control.

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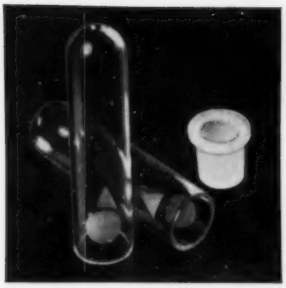

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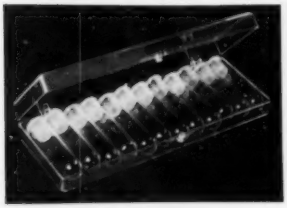

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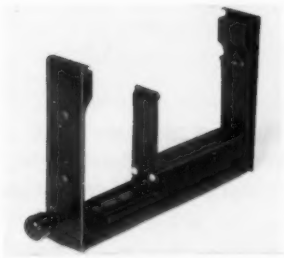

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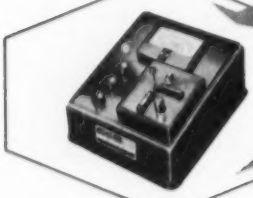

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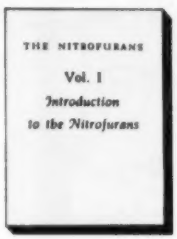

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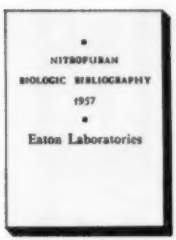

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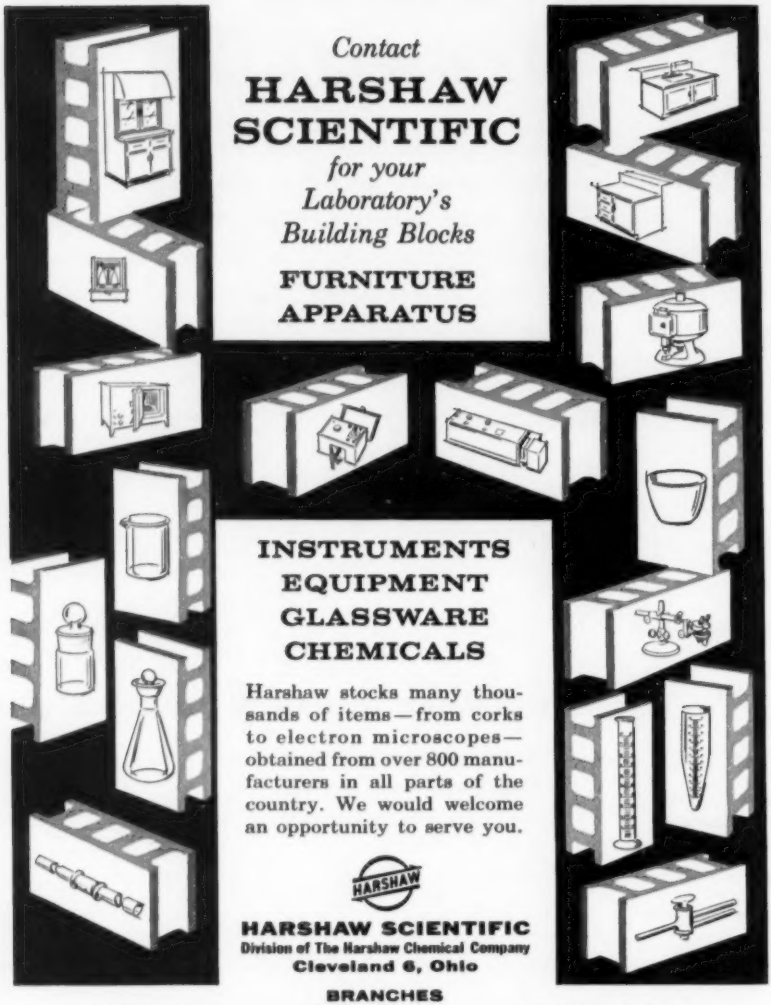

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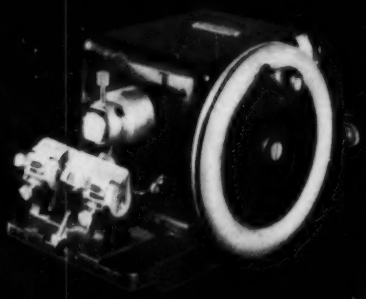

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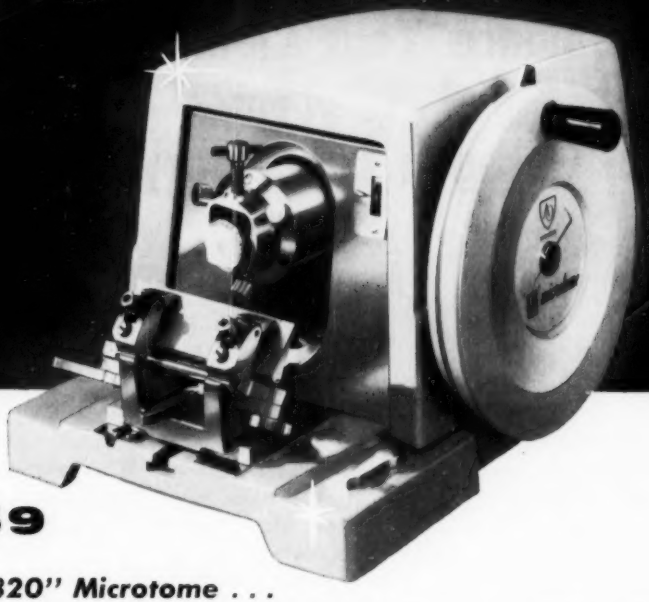

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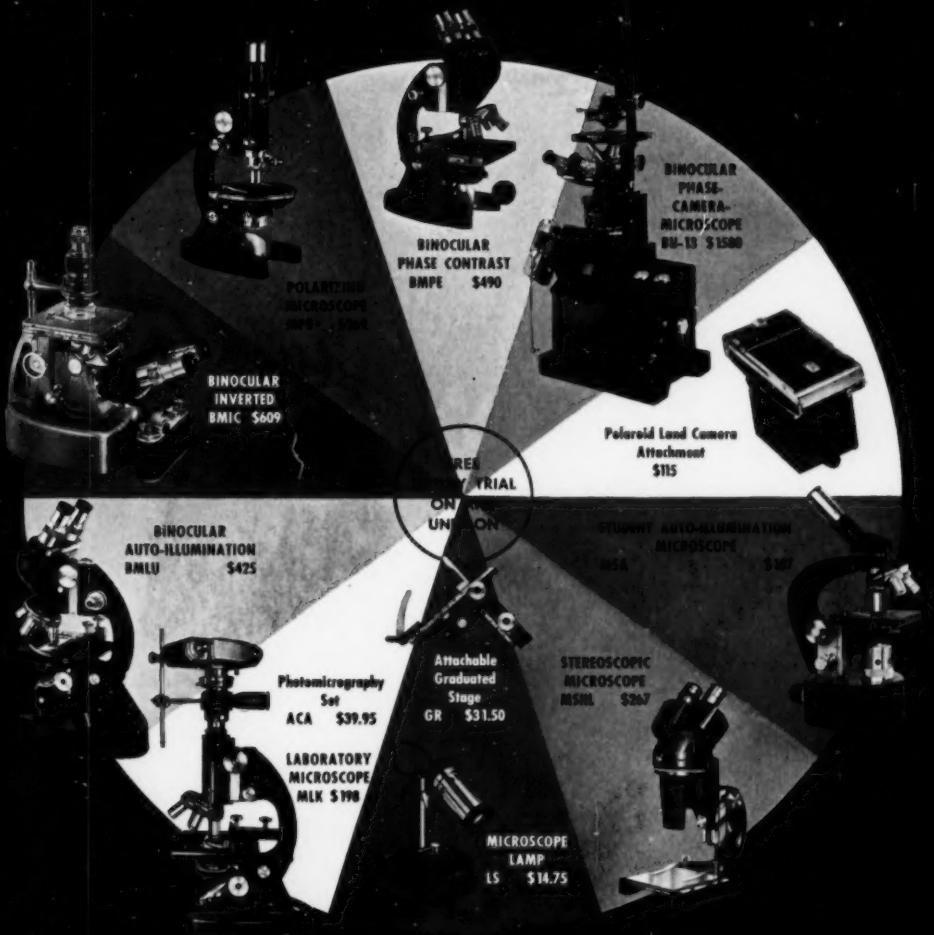
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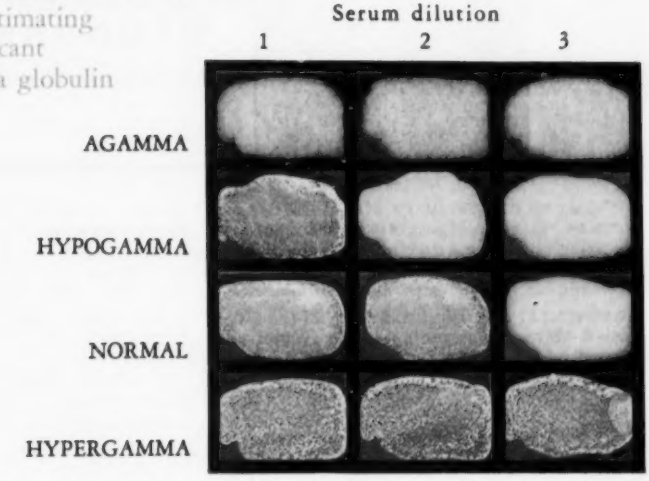

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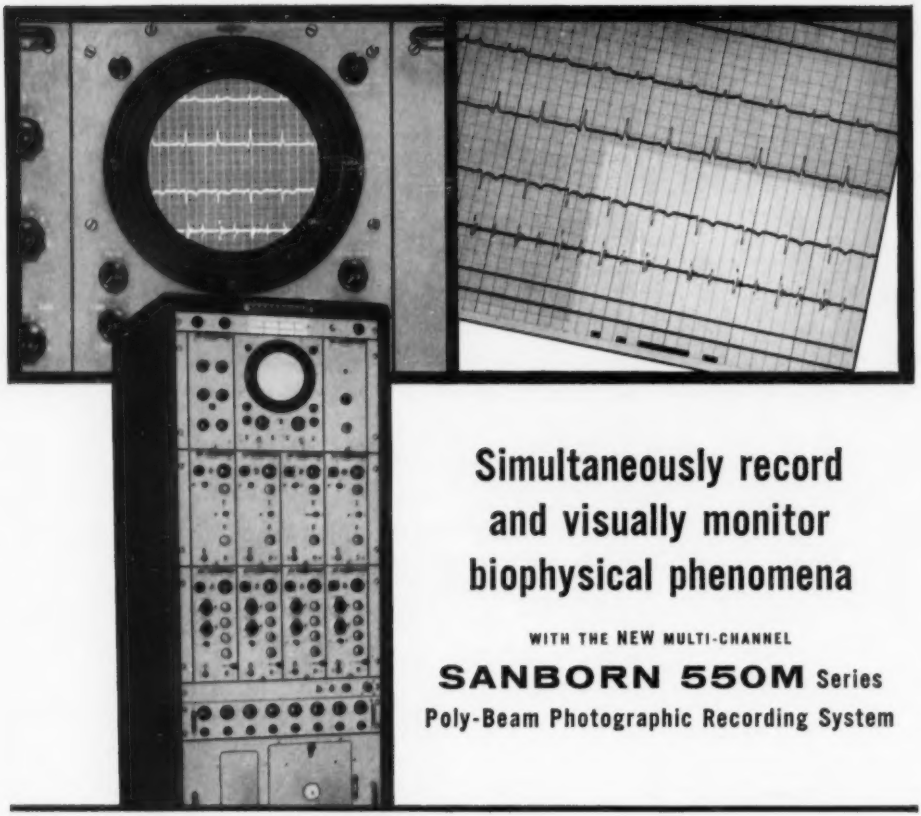

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The Journal of Laboratory and Clinical Medicine

VOL. 53 APRIL, 1959 No. 4

CLINICAL AND EXPERIMENTAL

EARLY AND LATE HEMODYNAMIC EFFECTS OF MITRAL COMMISSUROTOMY

WILLIAM S. LYONS, M.D., ROBERT G. TOMPKINS, M.D.,* JOHN W. KIRKLIN, M.D., AND EARL H. WOOD, M.D., PH.D.
ROCHESTER, MINN.

THE hemodynamic changes in the immediate postoperative period and short term following mitral commissurotomy have been reported by several authors.[1-6] An excellent long-term clinical study has been published by Ellis and Harken[7]; however, with two exceptions,[8, 9] published reports on the circulatory dynamics in the long-term postoperative period have been few and rather limited.[10, 11]

The patients constituting the series to be dealt with herein were the first 12 among those subjected to mitral commissurotomy during the period April, 1951, through October, 1952, who accepted right-heart catheterization 3 or more times and whose follow-ups have been extended 4½ to 6 years from the dates of operation. A standard technique of catheterization was used.[12, 13] Generally, the occasions were 1 week before commissurotomy, 3 weeks after (range 10 to 90 days), and 1 year after (range 8 to 30 months).

A brief summary of the hemodynamic data obtained has appeared elsewhere.[14] This paper is to present the complete data on these 12 patients, fully documenting the earlier statements concerning them, and to offer an analysis of the data.

From the Mayo Clinic and Mayo Foundation, Rochester, Minn.

A portion of this material is derived from a thesis submitted by Dr. Tompkins to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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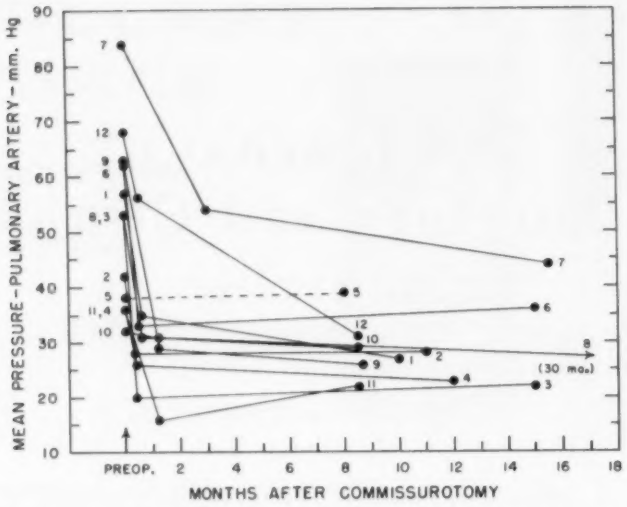

Fig. 1.—Pulmonary artery mean pressure at rest, before mitral commissurotomy, and in follow-up studies. The decrease after commissurotomy was consistent and the lower postoperative pulmonary artery pressure was maintained essentially unchanged through the rest of the period shown. The numerals designate the individual patients listed in Table I. The broken lines connect values for which corresponding early postoperative data were not obtained.

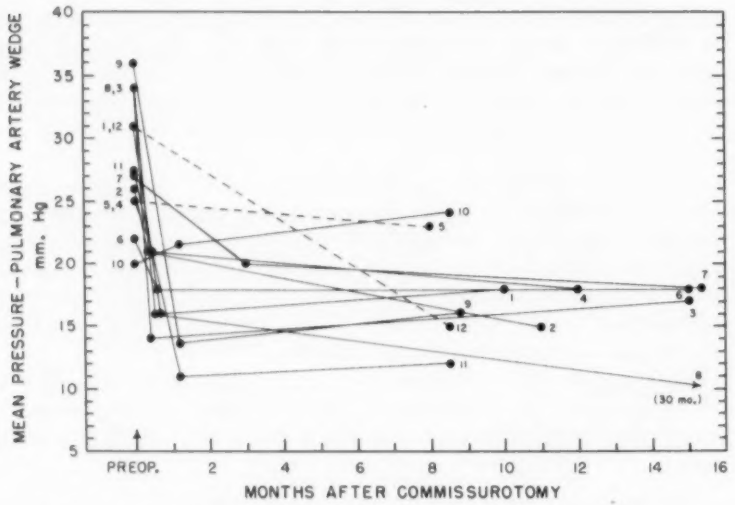

Fig. 2.—Pulmonary artery wedge pressure at rest, before mitral commissurotomy, and in follow-up studies. After the early postoperative decrease the wedge pressure remained practically unchanged through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

CLINICAL RESULTS

Along with the objective data, the clinical status of the 12 patients comprising the series deserves notice. Between the times of the preoperative and the second postoperative catheterizations, on the basis of the functional classification defined by the New York Heart Association, 5 patients improved from Group III to Group II, 6 from Group III to Group I, and 1 from Group IV to Group I. Thus, in each patient in this series surgical treatment brought improvement and, also, in no case had there been clinical deterioration between the first postoperative studies (at 10 to 90 days) and the second (at 8 to 30 months). Indeed, during the extension of the recovery period 9 patients had improved further in their functional classification or their condition had permitted further easing of restrictions on their activity.

Now, 4½ to 6 years after operation, all 12 patients are alive. Nine, for practical purposes, have sustained their maximal clinical improvement to the present time; 4 of them have reported further slight gains, 2 of them no change, and 3 of them slight losses. The clinical status of 3 other patients (Cases 6, 9, and 12) regressed significantly during the third to fifth years following operation, in one case after an illness strongly suggestive of rheumatic endocarditis. Cardiac recatheterization produced clear evidence that stenosis of the mitral valve had recurred in these 3 patients. Two have undergone reoperation, at which recurrent severe mitral stenosis was found.

OBJECTIVE DATA

The vital statistics and a summary of the hemodynamic data obtained in each patient when resting supine, when exercising, and when breathing 99.5 per cent oxygen are given in Table I. The exercise consisted of pedaling a bicycle ergometer from the supine position for periods of 3½ to 7 minutes.[13]

Pulmonary Artery Pressures.—

Mean pressure: In every case but one (Case 5), mitral commissurotomy was followed by a reduction in the resting pulmonary artery mean pressure. The average mean pressure was 52 mm. Hg before operation, 33 mm. Hg at 10 to 90 days after operation, and 29 mm. Hg at 8 to 30 months postoperatively. The lower postoperative pressures were maintained essentially unchanged for the duration of the follow-up period (Fig. 1). The level of pulmonary artery pressure during exercise was only slightly reduced, the average mean falling from 68 preoperatively to 56 at 10 to 90 days postoperatively and 58 at 8 to 30 months. Among all 9 cases in which the exercise data are complete, only one patient failed in the later postoperative study to maintain essentially the early postoperative improvement (Table I).

Wedge pressure: Preoperatively the resting mean wedge pressure averaged 28 mm. Hg. Ten to 90 days postoperatively this pressure was decreased 40 per cent to an average of 17, and at 8 to 30 months the average was 18 mm. Hg (Fig. 2). One patient (Case 10) failed to obtain a reduction in pulmonary

TABLE I. BASIC HEMODYNAMIC DATA ON 12 CASES OF MITRAL STENOSIS COLLECTED IN THE LONG-TERM

CASE	TIME OF STUDY	AGE AND SEX	N. Y. HEART GROUP	STATE	SURFACE AREA (M.²)	HEART RATE	OXYGEN CONSUMPTION (ML./MIN./M.²)	CARDIAC INDEX (L./MIN./M.²)
1	Preop.	52 M	III d	R	1.62	87‡	139	1.8
				Ex		103	184	1.8
	Postop. 21 days		II b	R	1.64	78‡	124	1.9
				Ex		99	223	2.3
	Postop. 10 mo.		II b	R	1.75	76‡	130	2.4
				Ex		108	312	2.8
2	Preop.	36 M	III d	R	1.92	54‡	114	1.5
				Ex		72	158	1.8
	Postop. 10 days		II b	R	1.79	49‡	124	2.2
				Ex		61	179	2.5
	Postop. 11 mo.		II b	R	1.92	60‡	127	2.2
				Ex		82	306	3.1
3	Preop.	27 F	III c	R	1.59	96	106	2.5
				Ex		120	305	3.0
	Postop. 12 days		II c	R	1.57	84	115	3.2
				Ex		135	135	4.1
	Postop. 15 mo.		I b	R	1.57	75	93	3.6
				Ex		130	415	4.8
4	Preop.	23 F	III c	R	1.53	80	143	3.9
				Ex		120	391	4.2
	Postop. 12 days		II c	R	1.49	90	137	3.8
				Ex		114	409	5.5
	Postop. 12 mo.		I b	R	1.56	92	129	3.4
				Ex		137	410	5.2
5	Preop.	33 F	III d	R	1.70	54‡	129	2.4
				Ex		92	353	2.8
	Postop. 3 wk.		II c	R	1.68	48‡	139	2.1
				Ex		108	388	3.7
	Postop. 8 mo.		I b	R	1.62	48‡	144	2.2
				Ex		87	362	3.3
6	Preop.	39 F	III d	R	1.36	106	157	3.3
				Ex		—	—	—
	Postop. 17 days		II c	R	1.34	84	142	3.0
				Ex		—	—	—
	Postop. 15 mo.		II b	R	1.30	76	138	2.9
				Ex		102	394	3.8
	Postop. 41 mo.		III c	R	1.30	72	134	2.8
7	Preop.	27 F	III c	R	1.58	64	137	2.3
				Ex		168	284	1.8
	Postop. 3 mo.		II c	R	1.54	60	133	2.8
				Ex		170	367	3.2
	Postop. 15½ mo.		II b	R	1.53	60	121	2.3
				Ex		149	386	3.2
8	Preop.	35 M	III d	R	1.71	80‡	172	3.0
				Ex		—	—	—
	Postop. 17 days		II b	R	1.69	68‡	132	2.4
				Ex		—	—	—
	Postop. 30 mo.		I b	R	1.77	60‡	139	2.3
				Ex		92	416	4.1

1 WEEK BEFORE COMMISSUROTOMY, IN THE EARLY POSTOPERATIVE PERIOD, AND POSTOPERATIVE FOLLOW-UP

PULMONARY ARTERY		RIGHT ATRIAL	VASCULAR RESISTANCE DYNE SEC. CM.$^{-5}$		MITRAL VALVE	BREATHING 99.5 PER CENT OXYGEN	
MEAN PRESSURE (MM. Hg)	MEAN WEDGE PRESSURE (MM. Hg)	MEAN PRESSURE (MM. Hg)	TPR*	PAR†	AREA INDEX (CM.^{2})	CARDIAC INDEX (L./MIN./M.^{2})	PULMONARY ARTERY MEAN PRESSURE (MM. Hg)
57	31	7	1,540	700	0.4	1.7	47
63	42	—	1,710	570			
35	16	5	900	490	0.7	1.9	32
55	22	—	1,170	550			
27	28	5	520	100	0.9	1.8	30
56	29	—	930	350			
42	26	6	1,130	430	0.4	1.5	40
67	—	—	1,570	—			
28	21	5	560	140	0.7	2.1	23
38	27	—	680	200			
28	15	8	520	150	0.8	2.1	30
39	26	—	530	180			
53	34	4	1,090	390	0.7	2.8	35
62	—	—	1,030	—			
20	14	3	320	100	1.6	2.8	18
44	34	—	430	130			
22	17	9	270	70	1.8	3.6	19
43	40	—	460	30			
36	25	4	490	150	1.5	2.7	33
47	39	—	570	100			
26	21	7	370	70	1.4	3.0	23
42	—	—	410	—			
23	18	3	370	80	1.5	2.3	42
44	28	—	430	50			
38	25	10	690	200	0.7	2.3	42
—	—	—	—	—			
—	—	23	—	—	—	—	—
—	—	—	—	—			
39	23	12	870	360	0.6	2.2	42
73	34	—	1,080	580			
62	22	4	1,130	730	1.1	2.3	48
—	—	—	—	—			
33	18	7	650	290	1.2	2.6	35
—	—	—	—	—			
36	18	3	700	470	1.1	—	—
68	27	—	1,100	670			
68	24	6	1,500	1,000	0.7	3.1	56
84	27	8	1,830	1,240	0.6	2.0	59
112	—	—	3,130	1,960			
54	20	6	990	620	0.9	—	46
113	—	—	1,800	—			
44	18	1	1,000	590	0.8	2.6	40
108	43	—	1,730	1,040			
53	34	9	610	290	0.8	2.2	47
—	—	—	—	—			
31	16	9	710	340	0.9	2.1	25
—	—	—	—	—			
26	10	4	510	310	1.5	2.4	18
43	26	—	470	190			

TABLE I—

CASE	TIME OF STUDY	AGE AND SEX	N. Y. HEART GROUP	STATE	SURFACE AREA (M.²)	HEART RATE	OXYGEN CONSUMPTION (ML./MIN./M.²)	CARDIAC INDEX (L./MIN./M.²)
9	Preop.	32 F	IV d	R	1.52	96	142	3.0
				Ex		120	214	3.0
	Postop. 5 wk.		II e	R	1.46	64	133	3.0
				Ex		102	348	4.5
	Postop. 9 mo.		I b	R	1.52	66	143	3.3
				Ex		112	452	4.8
	Postop. 58 mo.		II b	R	1.56	150‡	149	2.9
				Ex				
10	Preop.	42 F	III e	R	1.56	80	113	2.3
				Ex		93	244	3.3
	Postop. 5 wk.		II e	R	1.56	93	120	3.0
				Ex		126	339	4.5
	Postop. 8½ mo.		I b	R	1.62	76	115	2.4
				Ex		138	405	4.2
11	Preop.	38 F	III e	R	1.30	76	138	3.5
				Ex		130	389	4.3
	Postop. 5 wk.		II b	R	1.30	90	170	4.2
				Ex		114	338	4.1
	Postop. 8½ mo.		II b	R	1.35	96	135	3.4
				Ex		124	425	5.0
12	Preop.	31 F	III d	R	1.45	80	135	2.1
				Ex		114	262	2.4
	Postop. 16 days		II e	R	1.40	110	157	3.3
				Ex		124	388	4.9
	Postop. 8½ mo.		I b	R	1.49	72	126	2.5
				Ex		132	391	3.8
	Postop. 47 mo.		II e	R	1.50	88	138	2.4
				Ex			333	2.7
Averages								
	Preoperative (1 week)			R			135	2.6
				Ex			278	2.8
	Postoperative (10 to 90 days)			R			136	2.9
				Ex			311	3.9
	Postoperative (8 to 30 months)			R			128	2.7
				Ex			390	4.0

R = resting state; Ex = on exercise.
*TPR = total pulmonary resistance.
†PAR = pulmonary arteriolar resistance.
‡Atrial fibrillation occurred.

artery wedge pressure at rest or on exercise. The clinical improvement was very satisfactory, however, and a significant increase in cardiac output during exercise was demonstrable. With regard to the rise in wedge pressure with exercise, the comparative data are available on 5 patients before operation and 8½ to 12 months after operation. The average mean wedge pressure at rest for these 5 patients was 27 mm. Hg (range 20 to 31) preoperatively with a rise to 38 (range 29 to 42) on exercise. At determinations 8½ to 12 months postoperatively, the average mean wedge pressure at rest was 19 (range 12 to 28) with a rise to 30 (range 20 to 43) on exercise. Thus, the increase in

Cont'd

PULMONARY ARTERY		RIGHT ATRIAL MEAN PRESSURE (MM. Hg)	VASCULAR RESISTANCE DYNE SEC. CM.$^{-5}$		MITRAL VALVE AREA INDEX (CM.^{2})	BREATHING 99.5 PER CENT OXYGEN	
MEAN PRESSURE (MM. Hg)	MEAN WEDGE PRESSURE (MM. Hg)		TPR*	PAR†		CARDIAC INDEX (L./MIN./M.^{2})	PULMONARY ARTERY MEAN PRESSURE (MM. Hg)
63	36	5	1,100	540	0.7	3.6	48
67	—	—	1,160	—			
29	14	2	530	280	1.2	—	24
59	28	—	730	380			
26	16	2	400	160	2.0	2.7	20
50	—	—	500	—			
49	37	9	870	210	0.7	—	—
32	20	4	710	240	0.8	2.4	24
44	38	—	690	90			
31	22	9	540	150	1.2	—	21
59	41	—	670	210			
29	24	7	600	100	0.8	—	—
75	43	—	890	380			
36	28	2	640	150	0.9	3.0	30
65	29	—	930	510			
16	11	4	240	70	2.2	3.4	12
25	19	—	380	90			
22	12	3	380	180	1.7	3.5	15
34	20	—	400	40			
68	31	9	1,750	950	0.5	—	59
89	42	—	2,090	1,100			
56	—	7	950	—	—	3.6	43
65	—	—	770	—			
31	15	2	660	340	1.1	2.3	27
64	29	—	900	490			
64	35	9	1,420	640	0.5	2.4	56
81	39	—	1,620	840			
52	28		1,060	500	0.8	2.4	43
68	38		1,430	720			
33	17		610	260	1.2	2.7	27
56	29		780	260			
29	18		570	240	1.2	2.6	28
58	31		790	360			

the mean wedge pressure on exercise 8½ to 12 months postoperatively was rather closely proportionate to the preoperative increase. However, absolute values were substantially lower; so in 4 of the 5 patients the postoperative wedge pressure during exercise was approximately equal to or considerably lower than the preoperative value during rest.

Calculated Pulmonary Vascular Resistance.—At rest and during exercise the calculated total pulmonary resistance was reduced by an average of approximately 45 per cent in the immediate postoperative period; and although individual patients showed increases or decreases on restudy at 8 to 30 months after operation, the major portion of the large initial reduction was uniformly maintained (Fig. 3).

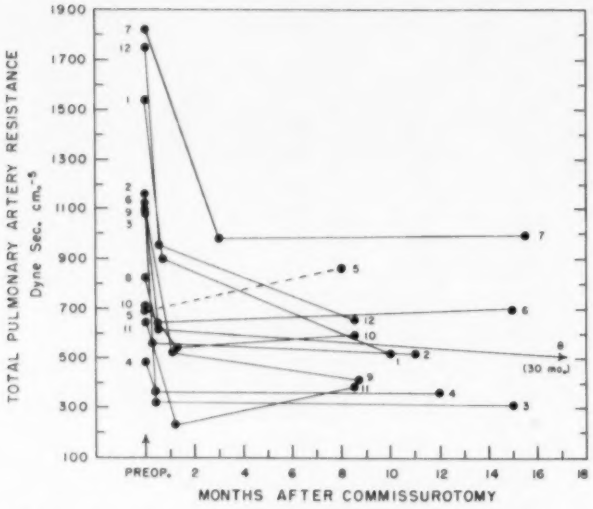

Fig. 3.—Total pulmonary artery resistance at rest, before mitral commissurotomy, and in follow-up studies. The total pulmonary vascular resistance decreased consistently and the lower postoperative values were maintained within the limits of error of such determinations through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

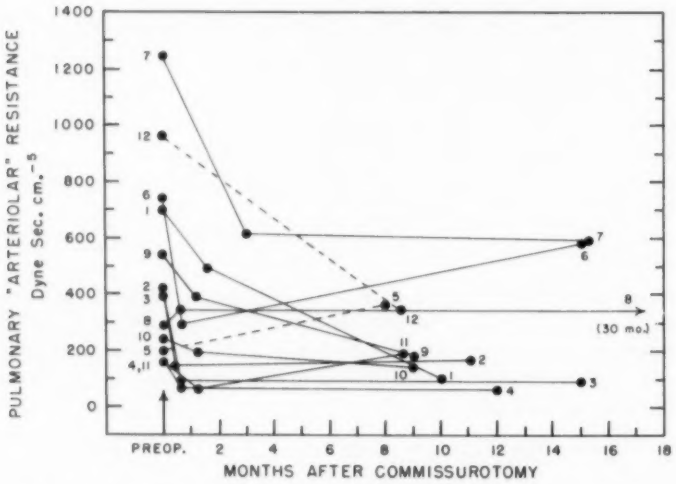

Fig. 4.—Pulmonary arteriolar resistance at rest, before mitral commissurotomy, and in follow-up studies. The lower postoperative values were generally maintained within the limits of error for such determinations through the rest of the period shown. (Details are explained in the legend for Fig. 1.)

In these 12 cases pulmonary arteriolar resistance comprised roughly half of the total resistance across the lesser circulation. The improvement with respect to this variable was proportional to the reduction in total pulmonary resistance (Fig. 4).

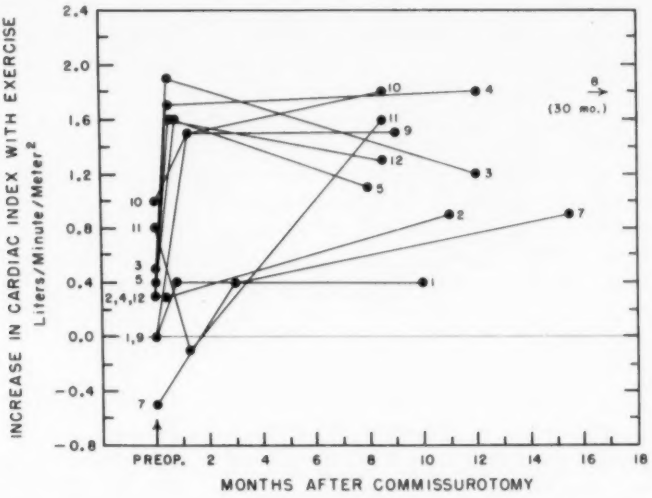

Fig. 5.—Changes in cardiac output with exercise, before mitral commissurotomy and in follow-up studies. The improvement in the increase of cardiac output with exercise was well maintained during the period shown. (Details are explained in the legend for Fig. 1.)

Cardiac Index During Rest and Exercise.—The normal cardiac index at rest has been given by Cournand[15] as 3.1 to 3.8 L./min./M.². The figures for healthy subjects studied[16] in this laboratory are 2.7 to 4.3 L./min./M.² with a mean increase on a standard form of exercise of 1.0 to 3.2 L./min./M.². Of the 12 patients in the present series, 8 showed a rise in the resting cardiac index postoperatively (Fig. 5). Six of these were patients whose preoperative index was subnormal, that is, below 2.7 L./min./M.² (Table I). Preoperatively, the 10 patients from whom the comparative data were obtained could increase their resting cardiac index by an average of only 12 per cent (310 ml./min./M.²) in response to exercise. In the early postoperative period they were able to increase it by 33 per cent, and 8 to 30 months postoperatively the entire series of 12 produced an average increase of 48 per cent. The absolute increase in response to exercise correlated well with the degree of clinical improvement (Fig. 6). At 8 to 30 months postoperatively the 7 patients who had improved from functional Class III or IV to Class I were able to increase their cardiac index above the resting level by 1.1 L./min./M.² or more, for an average increase of 1.5 L./min./M.². The 5 patients who had improved from Class III to Class II were able to increase their index by an average of 0.9 L./min./M.².

Stroke Index.—In healthy subjects, under the conditions of exercise in this laboratory, cardiac output is increased primarily through an increase in heart

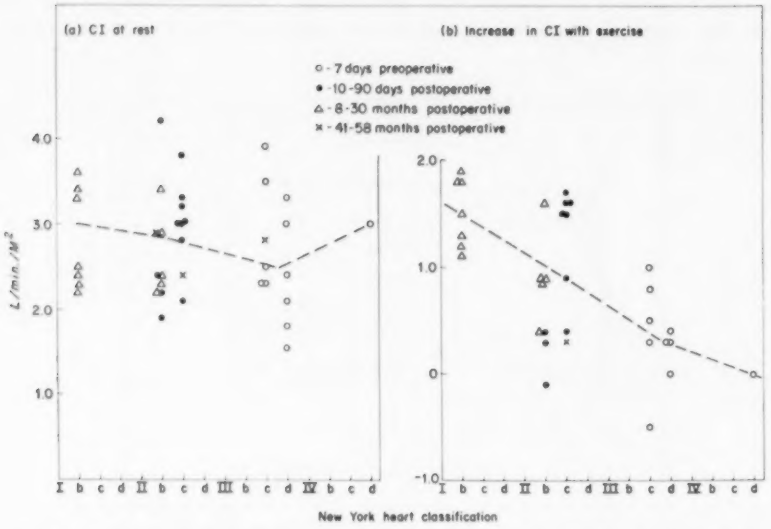

Fig. 6.—Relationship of cardiac output at rest (left panel) and its change with exercise (right panel) to the clinical status of the patients before and after mitral commissurotomy. The broken line shows the trend of average values. Between the cardiac output at rest and the clinical status of the patient, no correlation is apparent. However, the correlation of clinical status with the increase in the cardiac output associated with exercise is significant.

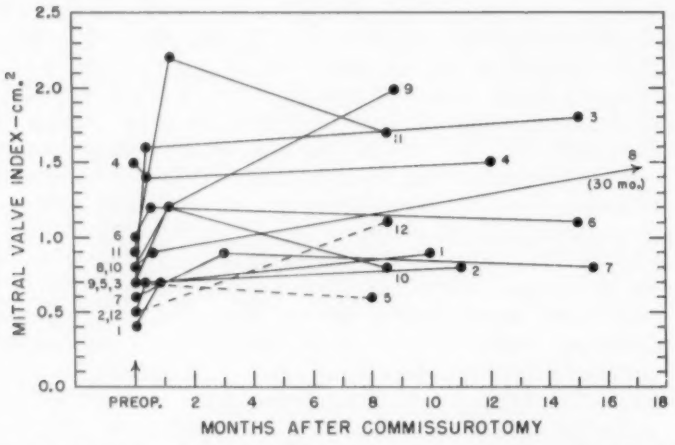

Fig. 7.—Mitral valve area index at rest, before mitral commissurotomy and in follow-up studies. Although there was no improvement with respect to this variable in several cases, neither was there significant deterioration during the rest of the period shown. (Details are explained in the legend for Fig. 1.)

rate and the change noted in the stroke index varies between limits of −16.7 to +14.7 ml./beat/min./M.^{2}. Only one patient of the present series showed a change in the stroke index outside this range of values preoperatively or postoperatively.

Mitral Valve Area Index.—The formula of Gorlin and Gorlin[17] was used in calculating the mitral valve area index. The theoretical limitations of the formula with reference to actual anatomic conditions are well known; however, it is of value as an index for comparison from patient to patient and particularly for comparison before and after operation in the same patient. In the series of 12 this index averaged 0.8 cm.^{2} preoperatively, 1.2 cm.^{2} 10 to 90 days postoperatively, and 1.2 cm.^{2} 8 to 30 months postoperatively (Fig. 7). Dexter[18] has stated that finger fracture may be expected to increase the mitral valve area by only 30 to 50 per cent. Although in this series the knife was used regularly to supplement finger fracture, the average increase in mitral valve area produced by commissurotomy was similar to that which Dexter observed. But results were not uniform: in 7 cases the calculated postoperative mitral area was twice the preoperative value, and in 5 cases it remained essentially unchanged (Table I).

Blood Pressure and Systemic Resistance.—In the 12 patients of this series the systemic blood pressure tended to be subnormal before commissurotomy; and the total systemic resistance* was consistently high. Normal values for this laboratory according to the data of Barratt-Boyes and Wood[16] are: systemic pressure, 135 systolic and 71 diastolic (standard deviations 15.7 and 7.4) with a mean of 91 (S.D. 8.7); and total systemic resistance, 1,130 ± 178 dyne sec. cm.$^{-5}$.

Preoperatively in this series of patients the pressures were: systolic 114 (range 93 to 139), diastolic 66 (range 58 to 86), and mean 82 (range 71 to 97). The average systemic pressures 10 to 90 days postoperatively were: systolic 124 (range 87 to 156), diastolic 65 (range 49 to 83), and mean 89 (range 62 to 104). These figures were practically unchanged at follow-up 8 to 30 months after operation.

The total systemic resistance averaged 1,580 dyne sec. cm.$^{-5}$ (range 965 to 2,470) preoperatively, 1,460 (range 940 to 2,540) at the early postoperative study, and 1,580 (range 1,060 to 2,160) 8 to 30 months postoperatively.

Response to Breathing High Oxygen Mixtures.—In the 12 patients the administration of 99.5 per cent oxygen for 6 minutes or more was followed by reduction in cardiac output in 18 of 31 comparative determinations. The average amount of these 18 reductions was 0.52 L./min./M.^{2} and the average of 8 rises was 0.26, 5 comparisons having been equal. The average change for all 31 was a fall of 0.24 L./min./M.^{2}. Studies on normal subjects in this laboratory have shown that the average fall in the cardiac index when breathing high oxygen mixtures is approximately 0.07 L./min./M.^{2}, which is not statistically significant.[16]

*Total systemic resistance was calculated by subtracting the mean right atrial pressure from the mean systemic arterial pressure.

The breathing of 99.5 per cent oxygen was associated with a fall in pulmonary artery mean pressure in 29 of 35 determinations. The average of the 35 changes was a fall of 6 mm. Hg and the average of the 29 falls was 8 mm. Hg.

Statistical analysis of the reduction in cardiac output and the fall in pulmonary artery mean pressure which resulted from breathing 99.5 per cent oxygen in this study reveals that these changes are of a high order of significance. At the present time the explanation for the reduction in cardiac output and pulmonary artery mean pressure is not entirely clear. Systemic resistance apparently rises slightly in response to high oxygen mixtures,[19] and the fall in pulmonary artery pressure is perhaps due to a reduction in pulmonary arteriolar resistance.

In these 12 patients the average change in the right ventricular work against pressure during the administration of 99.5 per cent oxygen, a reduction of 1.5 per cent, is small. However, it is of some clinical significance that in mitral disease, at least, this change in conjunction with the marked increase in oxygen tension in the arterial blood and the increase of approximately 2 volumes per cent in oxygen content may be expected to produce a significant improvement in the transport of oxygen to the body without increasing the work load on the myocardium.

COMMENT

The hemodynamic data available on these patients confirm the clinical impression that most of the improvement to be expected after mitral commissurotomy is likely to occur in the early postoperative period, within perhaps 3 to 4 weeks after operation. Further clinical recovery in the long-term postoperative period has been the experience of many, and the data from this study are consistent with this observation. However, the small gains noted between the early postoperative catheterizations and the recatheterizations performed 8 to 16 months postoperatively may be considered to fall within the limits of accuracy of the techniques employed. What is perhaps most important, the objective data obtained 8 to 30 months after operation indicate that the improvements which mitral commissurotomy produces in various hemodynamic variables are sustained through the long-term recovery period.

Value of Early Postoperative Measurements.—In a consideration of the possibility of recurrent stenosis, data from the early postoperative period are necessary for establishing on an objective basis the degree of relief actually produced by the operation. On the basis of objective data obtained before commissurotomy, soon after it, and in the long-term follow-up as well, it is possible to bring into proper perspective the emotional aspects of this therapy. Without question the psychologic impact of commissurotomy is great in many cases and is, of course, most influential in the early recovery period. The many hemodynamic studies from the early recovery period demonstrate that objective improvement, which usually is slight to moderate, is nevertheless real. In addition, restudy of the 12 patients in this series in the long-term recovery

period shows clearly that the hemodynamic gains recorded immediately after mitral commissurotomy remained relatively unchanged in the remainder of the period, 8 to 15 months following operation.

Dexter[18] has speculated on the probable lasting effects of this operation and has suggested that stenosis would not recur except with a recurrence of carditis. Werkö and co-workers[1] have stated that without information on the permanence of valvotomy, Group II cases should not be offered this operation —that considerations largely prophylactic do not justify commissurotomy. The data obtained in this study provide some answer to these problems, for it can be said certainly for the group, and with remarkable consistency for the individuals, that the hemodynamic improvement measured at the early postoperative study was maintained for a year or more following operation. These findings are consistent with the observation of Sancetta and co-workers,[9] who catheterized 11 patients before mitral commissurotomy and 6 weeks and 12 months after it, and found that in 7 patients without associated valvular lesions the hemodynamic improvements noted at 6 weeks were maintained to the time of the 12-month study.

Correlation of Objective Data With Clinical Status.—Pulmonary artery mean pressure has been thought the most reliable objective criterion of the severity and symptomatology of mitral stenosis, and many postoperative hemodynamic studies have been based on determinations of this factor and the wedge pressure only. However, Ellis and associates[4] found only poor correlation of pulmonary artery pressure with the degree of disability in cases without operation, and Janton and co-workers[20] noted only very general correlation between the fall in pulmonary artery pressure after commissurotomy and the degree of clinical improvement. Eliasch[21] found the average mean pulmonary artery pressure to be lowest in New York Heart Association Groups I and II, higher in Group III, and highest in Group IV; however, he also noted marked inconsistency in individual cases.

In this study the several physiologic variables determined before and after the operation have been compared with the New York Heart classification groupings of the patients (Figs. 6 and 8). Significant correlations were found between the clinical status of the patient and the pulmonary artery mean pressure, pulmonary artery mean wedge pressure, total pulmonary resistance, pulmonary arteriolar resistance, pulmonary venous resistance, mitral valve area index, and increase in cardiac index with exercise. No correlation was demonstrable between the clinical status of the patient and the resting cardiac index.

Perhaps the hemodynamic variable which usually correlates best with the general clinical status of the individual patient is the magnitude of the increase in cardiac output in response to effort. In this small series (33 comparative determinations), all increases of cardiac index recorded from patients while in Group I were of 1.1 L./min./M.^{2} or more (average 1.5), while no increase in the patients in Groups III and IV was greater than 1.0 L./min./M.^{2} (average 0.3). Patients in Group I, then, were able to increase their cardiac output in

the low normal range; and patients in Groups III and IV were not. As expected, patients in Group II occupied an intermediate position with respect to this variable and showed an average increase in cardiac index of 0.9 L./min./M.^{2} with several determinations low in the normal range and several well below it.

Although the level of the mean pulmonary artery pressure also shows good general correlation with the clinical classification of these patients, the variations in individual cases point out the desirability of obtaining both pressure and flow data in the evaluation of a given patient.

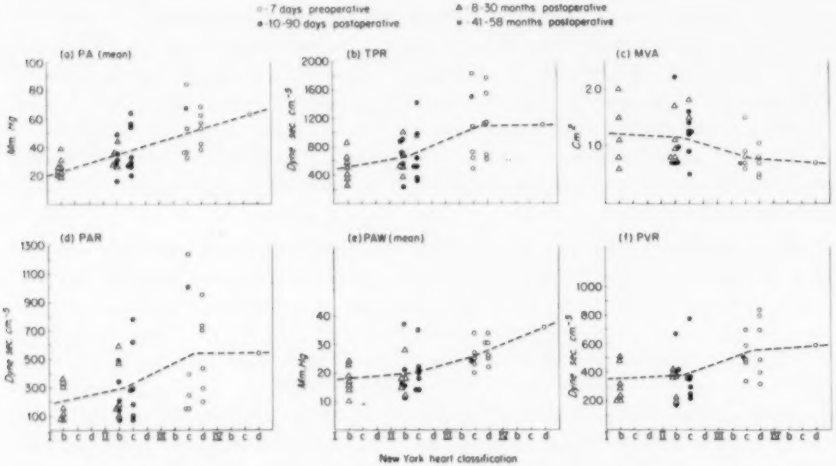

Fig. 8.—Relationship of hemodynamic parameters to the clinical status of the patients before and after commissurotomy. *a*, PA = pulmonary artery mean pressure. *b*, TPR = total pulmonary vascular resistance. *c*, MVA = calculated mitral valve area index. *d*, PAR = pulmonary arteriolar resistance. *e*, PAW = pulmonary artery mean wedge pressure. *f*, PVR = pulmonary venous resistance (TPR-PAR = PVR). The broken line represents the trend of average values within the 4 major classification groups. The correlation between these objectively determined hemodynamic variables and the clinical status of the patient is rough but apparently significant.

The direct effect of commissurotomy on the abnormal hemodynamic values present in mitral stenosis can be appreciated best by noting the degree of the reversion of these values toward normal in individual cases. Reference to Fig. 9, *a*, *b*, and *c* shows that in the present series of patients the postoperative decreases in the pulmonary artery wedge pressure, pulmonary artery mean pressure, and pulmonary arteriolar resistance are directly proportional to the degree of abnormality of these variables before operation. Thus, those patients with the highest pulmonary artery mean and wedge pressures and highest pulmonary arteriolar resistance before operation received the greatest absolute improvement with respect to these variables. Actually, these findings were unexpected, especially with regard to the pulmonary arteriolar resistance. High pulmonary arteriolar resistance has been thought by some to be a relative contraindication to commissurotomy, but the data from this series indicate that the greatest improvement in this variable may be expected in those cases with the most abnormal preoperative values.

Basis of Postoperative Change in Pulmonary Vascular Resistance.—The response of the pulmonary arteriolar resistance to mitral commissurotomy is of considerable theoretical interest. Other studies from this laboratory[22] in which left atrial and pulmonary artery pressures were taken in the operating room immediately before and after opening of the valve have demonstrated that the observed reduction in pulmonary artery mean pressure was closely similar in magnitude to the simultaneous reduction in left atrial pressure achieved by the commissurotomy. Although Fick determinations of cardiac output were not

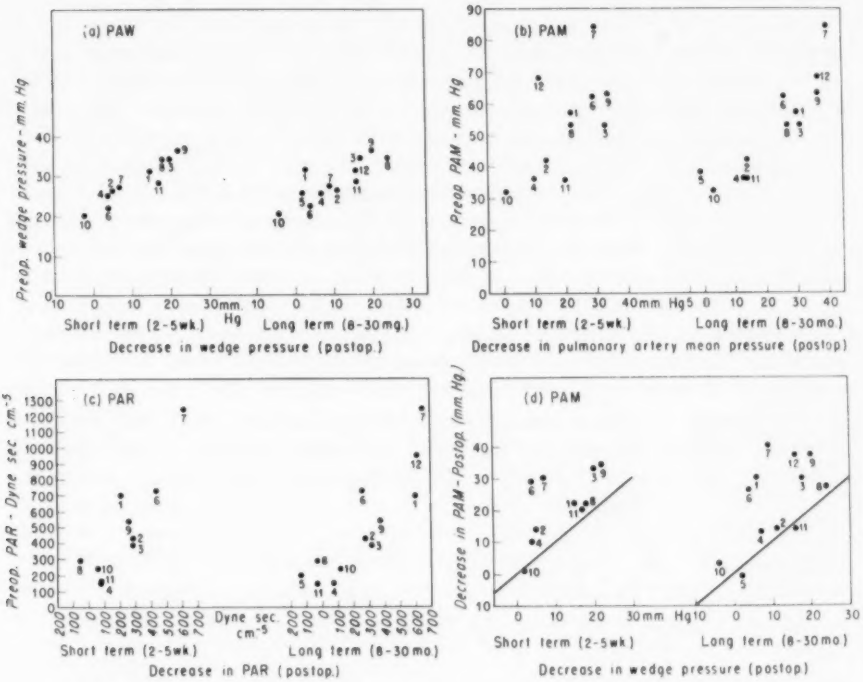

Fig. 9.—The relationship of preoperative values of pulmonary artery wedge pressure (*a*), pulmonary artery mean pressure (*b*), and pulmonary arteriolar resistance (*c*) to the decrease in these parameters following mitral commissurotomy. The decrease was directly proportional to their preoperative values. *d*, The relation of the decrease in pulmonary artery wedge pressure to the decrease in pulmonary artery mean pressure following mitral commissurotomy. If the decreases of each kind were equal, all points would fall on the diagonal lines. Since the decrease in pulmonary artery pressure exceeds that in the wedge pressure, more than a simple back-pressure relationship is involved in this effect. (The numerals indicate the individual patients listed in Table I.)

made simultaneously with the pressure recordings, the pressure pulse tracings indicate that cardiac output remained essentially the same for the two sets of pressure determinations. Thus the immediate reduction in pulmonary artery mean pressure as a result of valvotomy appears to be a simple back-pressure phenomenon.

A further reduction in pulmonary artery resistance, larger proportionately than the reduction in the wedge pressure (Fig. 9, *d*), was measured 10

to 90 days after commissurotomy and was found essentially unchanged on recatheterization 8 to 30 months postoperatively. Donald and co-workers[8] also observed that the reduction in the pulmonary artery pressure was greater than the reduction in the wedge pressure 2 years after mitral commissurotomy. It appears, therefore, that the high pulmonary arteriolar resistance in mital stenosis is due at least in part to vasoconstriction, and also that it is partially reversible.

Comparison With Postoperative Effects Reported by Others.—Donald and co-workers[8] have reported recently the hemodynamic findings in 28 patients before successful mitral commissurotomy and 20 months after it. They found that the most consistent hemodynamic effect of the operation was a reduction in the resting cardiac index and a substantial reduction in the pressure work of the right ventricle. The data from the present study also show the reduction in pressure work. However, with regard to the resting cardiac index no consistent fall was found. On the contrary, the majority of patients in this series showed a rise in the resting cardiac index after operation, the rise tending most strongly to occur in those patients whose resting index was subnormal before operation (Table I). With regard to the several other variables determined in studies of this type, the data from the present investigation show a significant correlation with the clinical status of the patient as defined by the New York Heart classification (Figs. 6 and 8). In the study by Donald and associates[8] this relationship was less apparent, perhaps because a number of patients with known combined valvular lesions were included.

Recurrence of Mitral Stenosis.—The problem of recurrent mitral stenosis has been reviewed recently by Belcher.[23] He found opinions varying from a categorical denial of its occurrence to the belief that it occurred only after a recrudescence of carditis or after inadequate valvotomy. He reported 4 cases of clinical recurrence of stenosis in which these factors were thought to have been excluded. Objective hemodynamic evidence demonstrating, first, the improvement effected by successful operation and, second, the regression to the preoperative status, is scarce. Three patients (Cases 6, 9, and 12) in this series presented with clinical evidence of recurrence of stenosis in the third to fifth years following commissurotomy. All 3 had been catheterized 1 week preoperatively and recatheterized 16 to 35 days and 8 to 15 months postoperatively. The recurrence of stenosis was confirmed when they were again recatheterized 41 months to 58 months postoperatively. The 4 sets of data (Table I) demonstrate in an objective way the hemodynamic improvement effected by commissurotomy, the maintenance of this improvement for at least 8 to 15 months postoperatively, and the subsequent recurrence of stenosis in the fourth to fifth years following operation. As mentioned, 2 of these patients have been re-explored and found to have severe mitral stenosis. The third patient has not undergone re-exploration as yet.

Accuracy of Hemodynamic Measurements.—The relatively small degree of change between the immediate and later postoperative values determined by

cardiac catheterization is within the suspected margin of error of these measurements in most instances. This degree of reproducibility suggests that the actual error in the measurements was small. On the other hand, the relatively small change produced by commissurotomy in several of the hemodynamic variables indicates that those parameters must be determined with considerable accuracy in order to demonstrate consistently the objective improvement produced by successful operation. The need for both pressure and flow data in the preoperative and postoperative hemodynamic evaluation of these patients is reemphasized.

SUMMARY

Hemodynamic data are reported from 12 patients having had right-heart catheterization 1 week before, 10 to 90 days after, and 8 to 30 months after satisfactory mitral commissurotomy. The data demonstrate that the initial clinical and objective improvement following commissurotomy was sustained for at least 8 to 30 months in each of these patients. Although every patient in this series obtained definite clinical and objective hemodynamic benefit following mitral commissurotomy, each had some degree of residual stenosis after the operation. All cases have been followed up. All the patients are living 4½ to 6 years after operation, and the clinical improvement has been maintained by 9.

However, evidence both clinical and objective (from recatheterization and operation) of the recurrence of the preoperative stenotic condition was found in 3 of the cases 3 to 5 years after commissurotomy. Recurrence of stenosis cannot be clearly proved without cardiac catheterization soon after operation to establish on an objective basis the actual degree of relief of stenosis obtained through the operative procedure. Both accuracy and completeness are essential in these measurements. When the measured and derived data from determinations of pressure and of flow are compared with the clinical status of the 12 patients of this series before and after operation, significant correlation is apparent.

This study was made possible by the active participation of many clinical and laboratory colleagues. The assistance of Drs. B. Barratt-Boyes and H. J. C. Swan, Mrs. Jean Frank, and Miss Lucille Cronin was of particular importance.

REFERENCES

1. Werkö, L. Björck, G. Crafoord, C., Wulff, H., Krook, H., and Eliasch, H.: Pulmonary Circulatory Dynamics in Mitral Stenosis Before and After Commissurotomy, Am. Heart J. **45**: 477-490, 1953.
2. McAllister, F. F., Fitzpatrick, H. F., Powers, S. R., Jr., Papper, E. M., and Krahl, E.: Hemodynamic Studies Made During Mitral Valve Surgery, Proc. Surg. Forum, Clin. Congress, Am. Coll. Surgeons, Philadelphia, 1953, W. B. Saunders Company, pp. 312-316.
3. Moscovitz, H. L., Gordon, A. J., Braunwald, E., Amram, S. S., Sapin, S. O., Lasser, R. P., Himmelstein, A., and Ravitch, M. M.: Use of Simultaneous Left Heart Pressure Pulse Measurements in Evaluating the Effects of Mitral Valve Surgery, Am. J. Med. **18**: 406-414, 1955.
4. Ellis, L. B., Bloomfield, R. A., Graham, G. K., Greenberg, D. J., Hultgren, H. N., Kraus, H., Maresh, G., Mebane, J. G., Pfeiffer, P. H., Selverstone, L. A., and Taylor, J. A.: Studies in Mitral Stenosis. I. A Correlation of Physiologic and Clinical Findings, A. M. A. Arch. Int. Med. **88**: 515-531, 1951.

5. Varco, R. L., and Baronofsky, I. D.: Surgical Problems in Rheumatic Valvular Heart Disease, Journal-Lancet **72**: 545-554, 1952.
6. Wulff, H. B., Björck, G., Bergh, N. P., Krook, H., Axen, O., and Lundskog, O.: Studies in Mitral Stenosis. I. Results of One Year's Series of Surgically Treated Cases, Acta med. scandinav. **144**: 274-283, 1952.
7. Ellis, L. B., and Harken, D. E.: Factors Influencing Late Results of Mitral Valvuloplasty for Mitral Stenosis, Ann. Int. Med. **43**: 133-142, 1955.
8. Donald, K. W., Bishop, J. M., Wade, O. L., and Wormald, P. N.: Cardio-respiratory Function Two Years After Mitral Valvotomy, Clin. Sc. **16**: 325-350, 1957.
9. Sancetta, S. M., Mueller, R. P., and Gillespie, D. G.: Preoperative, Six-Week and Twelve-Month Postoperative Hemodynamic Studies in Patients Undergoing Mitral Commissurotomy, J. Lab. & Clin. Med. **42**: 945, 1953.
10. Husfeldt, E., Engell, H. C., and Pedersen, A.: Valvulotomy for Mitral Stenosis, Acta chir. scandinav. **105**: 144-160, 1953.
11. Carlotti, J., Joly, F., Sicot, J. R., and Voci, G.: Le rétrécissement mitral et son traitement par commissurotomie: Étude hémodynamique pré et post-opératoire du rétrécissement mitral, Semaine hôp. Paris **29**: 2079-2087, 1953.
12. Wood, E. H.: Special Instrumentation Problems Encountered in Physiological Research Concerning Heart and Circulation in Man, Science **112**: 707-715, 1950.
13. Wood, E. H.: Symposium on Cardiac Catheterization: Special Technics of Value in Cardiac Catheterization Laboratory, Proc. Staff Meet., Mayo Clin. **28**: 58-64, 1953.
14. Ellis, F. H., Jr., Kirklin, J. W., Parker, R. L., Burchell, H. B., and Wood, E. H.: Mitral Commissurotomy: An Over-all Appraisal of Clinical and Hemodynamic Results, A. M. A. Arch. Int. Med. **94**: 774-784, 1954.
15. Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr.: Measurement of Cardiac Output in Man Using the Technique of Catheterization of the Right Auricle or Ventricle, J. Clin. Invest. **24**: 106-116, 1945.
16. Barratt-Boyes, B. G., and Wood, E. H.: Cardiac Output and Related Measurements and Pressure Values in the Right Heart and Associated Vessels, Together With an Analysis of the Hemodynamic Response to the Inhalation of High Oxygen Mixtures in Healthy Subjects, J. Lab. & Clin. Med. **51**: 72-90, 1958.
17. Gorlin, R., and Gorlin, S. G.: Hydraulic Formula for Calculation of Area of Stenotic Mitral Valve, Other Cardiac Valves, and Central Circulatory Shunts, Am. Heart J. **41**: 1-29, 1951.
18. Dexter, L.: Pathologic Physiology of Mitral Stenosis and Its Surgical Implications, Bull. New York Acad. Med. **28**: 90-105, 1952.
19. Whitehorn, W. V., Edelmann, A., and Hitchcock, F. A.: Cardiovascular Responses to Breathing of 100 Per Cent Oxygen at Normal Barometric Pressure, Am. J. Physiol. **146**: 61-65, 1946.
20. Janton, O. H., Glover, R. P., O'Neill, T. J. E., Gregory, J. E., and Froio, G. F.: Results of the Surgical Treatment for Mitrol Stenosis: Analysis of One Hundred Consecutive Cases, Circulation **6**: 321-333, 1952.
21. Eliasch, H.: The Pulmonary Circulation at Rest and on Effort in Mitral Stenosis, Scandinav. J. Clin. & Lab. Invest. **4** (Suppl. 4): 13-99, 1952.
22. Lev, R., Connolly, D. C., Kirklin, J. W., and Wood, E. H.: The Immediate Hemodynamic Effects of Mitral Commissurotomy During Surgery, Proc. Surg. Forum, Clin. Congress, Am. Coll. Surgeons, Philadelphia, 1953, W. B. Saunders Company, pp. 13-17.
23. Belcher, J. R.: Restenosis of the Mitral Valve, Brit. Heart J. **20**: 76-82, 1958.

THE DETERMINATION OF THE MOLECULAR WEIGHT OF DIFFERENT AUTOPRECIPITINS IN HASHIMOTO'S DISEASE BY A NEW IMMUNOLOGIC METHOD

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ANTIBODIES are known to differ in molecular weight. The two most frequently described are those with molecular weights of 160,000 and 1,000,000[1]; the sedimentation constants of these antibodies are 7S and 19S, respectively. Some species, like the horse, produce precipitins of either molecular weight against certain antigens whereas rabbit precipitins seem to be of the 7S variety, although some hemolysins have sedimentation constants of 19S.[1] A human precipitin with a sedimentation constant of 19S has recently been described.[2]

The present communication will describe a new and simple immunologic method for the determination of the molecular weight of human precipitins. Application of this method to several sera from patients with Hashimoto's disease has shown that autoprecipitins against a highly purified thyroglobulin have molecular weights of either 160,000 or 1,000,000. Moreover, this study has contributed new data on the nature of these autoprecipitins and the antigenic structure of thyroglobulin.

MATERIALS AND METHODS

Human Sera.—The studies for the molecular weight determinations were performed with 4 sera from patients with proved Hashimoto's struma.

Rabbit Antihuman Gamma Globulin Serum.—Rabbit serum, which contained antibodies against both 7S and 19S components of human gamma globulin, has been described.[3] Some of this antiserum was rendered specific for 19S globulin by absorptions with 7S gamma-2 globulin.[3]

Human Thyroglobulin.—Normal human thyroid was homogenized in saline (40 Gm./100 ml.) and the insoluble material was removed by centrifugation. The soluble material was dialyzed against distilled water, lyophilized, and redissolved in distilled water. The thyroglobulin was subsequently twice precipitated by the addition of 3.5 M phosphate to a final concentration of 1.75 M phosphate[4] at 20° C. The slightly reddish precipitate was dialyzed against distilled water, lyophilized, and subsequently subjected to zone electrophoresis on starch. (Phosphate buffer pH 7.6; $\Gamma/2 = 0.1$; 3° C.) The central portion under the main peak was eluted with saline, dialyzed against distilled water, and lyophilized.

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This investigation was supported by a research grant (C-2359 M&I) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

Received for publication June 30, 1958.

This product, which was slightly yellow when in solution, was analyzed in the Spinco Model E ultracentrifuge and by paper electrophoresis. In the ultracentrifuge, it sedimented very similarly to another preparation prepared in this institute by phosphate precipitation,[4] i.e., the bulk of the protein sedimented as a tall sharp peak with a sedimentation constant of about 17S; two minor components which sedimented slower and faster than the main component were also present. On paper electrophoresis (phosphate buffer, pH 7.6; $\Gamma/2=0.1$) it migrated with a mobility between that of alpha-1 and alpha-2 globulin. The yellow color of this thyroglobulin preparation remained after two subsequent precipitations in 1.75 M phosphate. There was no apparent antigenic alteration since the final product reacted in an identical way as the thyroglobulin of the original thyroid extract; the purified product was a good antigen in the rabbit.[5]

Ultracentrifugal Fractionation of Hashimoto's Sera.—Hashimoto's sera I, II, and IV were subjected to ultracentrifugation in a partition cell at 59,780 r.p.m., as described previously.[6] The run was terminated after the 19S peak had passed through the partition. The contents of both the upper and lower compartments were analyzed for the presence of autoprecipitins.

THEORY

Human gamma globulin is composed of at least two types of molecules: one with a molecular weight of 160,000, the other with a molecular weight of 1,000,000. These two proteins have a few antigenic groupings in common[3] but are otherwise immunologically distinct and can be identified by the double gel diffusion method even when both are present.[3] The reagent, antiserum against gamma globulin, is easily produced in rabbits, and the two antigens can be easily identified by the characteristic curvatures of the precipitin lines.[8]

Let us assume that the autoprecipitins against thyroglobulin are gamma globulins of either the 7S or 19S variety (or both). Consequently, we would have a system in which these proteins would react with a rabbit antigamma globulin serum as well as with a human thyroglobulin. Let us therefore design the following experiment: in one reservoir of an Ouchterlony plate we place the rabbit antigamma globulin serum, and in the other two reservoirs we place thyroglobulin and Hashimoto's serum, respectively. The patterns of the precipitin lines, depending on whether the 7S or 19S globulin is the autoprecipitin, are illustrated in Diagram 1, *a* and *b*. In both instances it is assumed that only a fraction of either the 7S or 19S globulin is capable of reacting with thyroglobulin. If the autoprecipitin is a 7S globulin, the line formed by it and the thyroglobulin will cross the line formed by the 19S globulin and continue until it reaches the line formed by 7S globulin and the antigamma globulin serum. Since both the thyroglobulin and the antigamma globulin react with the same molecular species, 7S globulin, the two lines will coalesce. If all 7S globulins were antithyroglobulin, the lines would coalesce completely; however, since most of the 7S globulin is inert toward thyroglobulin, it will react with antigamma globulin serum only and a spur will be formed at the site of fusion for similar reasons as described previously,[9] i.e., the inert 7S globulin will diffuse through the precipitate formed by the reactive 7S and the thyroglobulin; once beyond that precipitate the inert 7S will be precipitated by the anti-7S of the rabbit antiserum (Diagram 1, *a*). However, if the antithyroid activity resided in the

19S globulins, then the line formed by Hashimoto's serum and the thyroglobulin would be deflected by the line formed between 19S and the rabbit antiserum (Diagram 1, *b*).

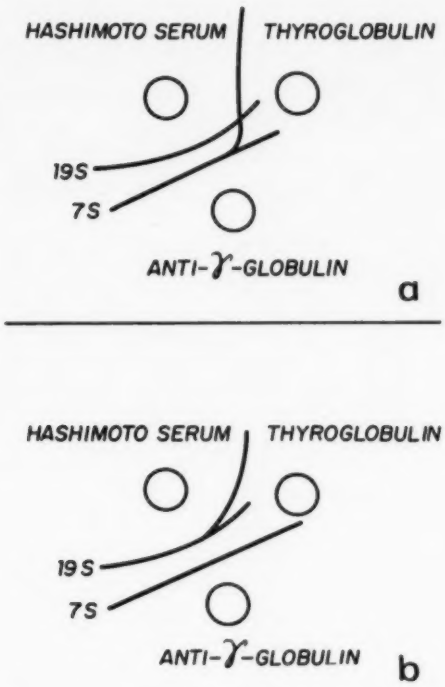

Diagram 1.—The theoretical precipitin patterns to be expected with Hashimoto's sera containing either (a) 7S or (b) 19S precipitins and reacting with thyroglobulin and antibodies against 7S and 19S gamma globulins.

RESULTS AND DISCUSSIONS

All the sera studied differed in their immunologic properties toward thyroglobulin (Fig. 1). Serum I was extremely potent and still produced one strong precipitin line at a dilution of 1:12. Serum II (Cup D) produced two lines. That this was not an artifact is apparent from the following evidence: (1) The two lines were produced in the region of equivalence, i.e., in the region in which the precipitate remains stationary. The production of multiple zones of precipitate with homogenous systems occurs only in the regions of antigen or antibody excess.[10] (2) Serum III (Cup C) produced one line only which coalesced with the line closest to the thyroglobulin reservoir (Fig. 1). The antigens responsible for the two lines formed with serum II seem to be antigenically related because both coalesced with the single line formed by serum I (Cup B); the resulting pattern is identical to the one previously described for macroglobulins by Korngold and Van Leeuwen,[11] (Diagram 1) and can probably be

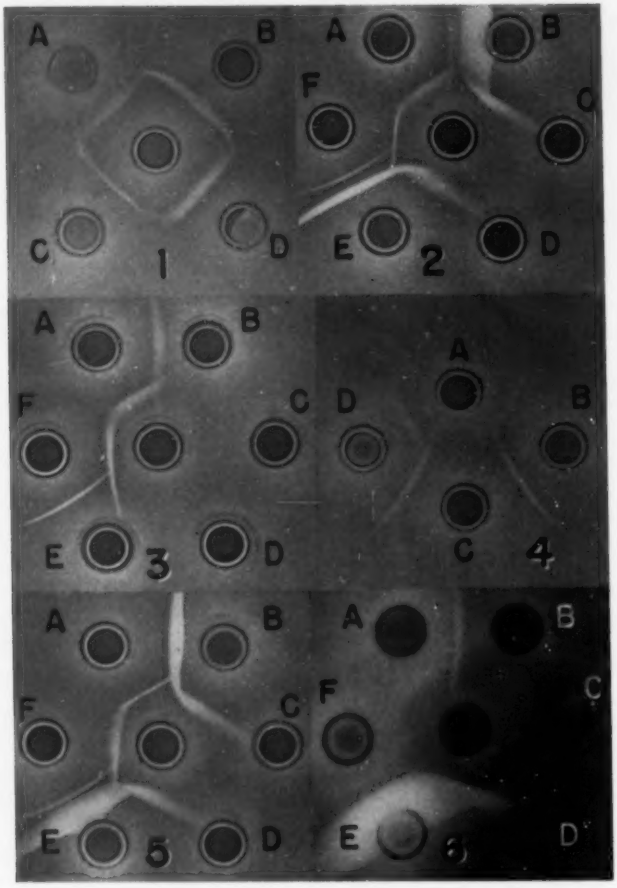

Fig. 1.—Center = Thyroglobulin (7.5 mg./ml.). A = Serum IV. B = Serum I (1:6). C = Serum III. D = Serum II.

Fig. 2.—Center = Thyroglobulin (5 mg./ml.). A = Serum II. B = Anti-γ-globulin serum. C and D = Empty. E = Anti-γ-globulin serum. F = Serum I (1:6).

Fig. 3.—Center = Thyroglobulin (5 mg./ml.). A = Serum II. B = Anti-γ-globulin serum adsorbed with 7S gamma-2 globulin. C and D = Empty. E = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. F = Serum I (1:4).

Fig. 4.—A = Thyroglobulin (5 mg./ml.). B = Serum III C = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. D = Serum IV.

Fig. 5.—Center = Thyroglobulin (5 mg./ml.). A = Serum I, top compartment of partition cell (1:3). B = anti-γ-globulin serum. C and D = Empty. E = Anti-γ-globulin serum. F = Serum I, bottom compartment of partition cell (1:3).

Fig. 6.—Center = Thyroglobulin (5 mg./ml.). A = Serum IV, bottom compartment of partition cell (1.2). B = Anti-γ-globulin serum absorbed with 7S gamma-2 globulin. C and D = Empty. E = anti-γ-globulin serum. F = Serum IV, top compartment of partition cell. (This plate was treated with Congo red in order to sharpen the contrast between the precipitin lines and the background.)

interpreted in the same manner: i.e., thyroglobulin contains two antigenically related but not identical components; serum I contains antibody against antigenic groupings they both have in common; serum II contains antibody against both common and specific groups, whereas serum III contains antibody specific for one of the components only. That sera from different patients may give rise to either one or two lines has been reported by others,[2, 12] and the pattern produced by serum II is identical to that published by Doniach and associates in their classic paper.[12] Serum IV (Fig. 1, Cup A) also produced one line that coalesced with the lines produced by the other sera. The 4 sera also differed in their potency in that serum I, even at a dilution of 1:6, produced a line that was visible within 24 hours. The lines produced by sera II and III took 2 to 3 days to appear and the sera lost all reactivity if diluted more than 1:4, whereas serum IV could not be diluted more than twofold.

Pressman and co-workers used the late appearances of the precipitin line, as compared with an egg albumin-rabbit anti-EA system, as additional evidence that the precipitin of their Hashimoto's serum was a macroglobulin. However, it should be noted that the time at which the line appears depends on several factors: the strength of the antiserum, the diffusion coefficients of both antibody and antigen, and the concentration of the latter. Since thyroglobulin is a large molecule with a small diffusion coefficient the line would appear later with a 7S antibody than would the line formed with egg albumin, which is a small molecule with a high diffusion coefficient, even if both reactions involved equal amounts of antibody.

The curvature of the lines obtained with sera I, II, and III was toward the antigen (thyroglobulin) reservoir, which suggests that the antigen diffuses more slowly than the antibody.[8] If we could be sure which of the 3 components with different sedimentation constants were the reactive antigens of thyroglobulin, an estimate of the antibody's molecular weight could be made. For example, if the 19S thyroglobulin were the reactive antigen, then the antibody was probably the 7S globulin. However, if the component greater than 19S were the precipitin, the antibody could be either 7S or 19S. (As will be shown later, the component with a sedimentation constant less than 19S is gamma globulin.)

The precipitin line formed by serum IV and thyroglobulin curved away from the antigen reservoir (Figs. 1 and 4), suggesting that the antibody diffused more slowly than did the thyroglobulin.[8] The diffusion coefficients of gamma-1 macroglobulin and thyroglobulin are approximately 1.8 and 2.6, respectively,[1] which agrees with the observed curvature of the precipitin lines. The differences in curvature of the lines produced by sera from patients with Hashimoto's struma is illustrated in Figs. 1 and 4. In this study, where the physical characteristics of the antigen are known, it is possible to obtain information about the nature of the antibodies from the curvature of the precipitin lines. However, for other less well-defined systems it will be essential to employ the method described above. The results with this immunologic method strongly indicate that the autoprecipitins of sera I, II, and III are 7S gamma globulins.

For example, from Figs. 2 and 3 it can be seen that the lines formed by thyroglobulin and Hashimoto's sera I, II, and III intersect the line formed by the 19S gamma globulin of Hashimoto's serum with antigamma globulin. This lack of interference between the two lines is even more apparent in Fig. 3 where the antigamma globulin serum was adsorbed with 7S gamma globulin. The pattern of the lines in Figs. 2, 3, and 5 resembles that of Diagram 1, *a*, and consequently one may conclude that the thyroglobulin reacts with 7S autoprecipitin rather than with 19S globulin. The use of undiluted Hashimoto's sera (Fig. 2, Cup A; Fig. 6, Cup F) resulted in very broad zones of precipitate owing to the high concentration of 7S gamma globulin, this being in extreme antigen excess relative to the antigamma globulin serum. Such zones have been described by others.[13]

The fact that the unadsorbed antigamma globulin serum also reacts with a component of the thyroglobulin preparation (Figs. 2 and 5) and that the resulting line coalesces with the line formed by the 7S gamma globulin of Hashimoto's serum indicates that the thyroglobulin is still contaminated with some gamma globulin as was pointed out above. It has been found by others[14] that molecules immunologically indistinguishable from 7S gamma globulin can be found in the alpha-2 globulin fraction; however, it is of some interest that we have been unable to detect this component in alpha-2 globulin isolated from serum by zone electrophoresis on starch, even in concentrations of 10 mg. per milliliter. From Fig. 4 it can be seen that the line formed by Hashimoto's serum IV (Cup D) and thyroglobulin (Cup A) never crossed the line formed by the 19S globulin and antigamma globulin serum (Cup C). Since the pattern is the same as pictured in Diagram 1, *b*, it may be concluded that the autoantibody of serum IV is a 19S gamma globulin similar to the one described by Pressman and co-workers.[2] The same plate (Fig. 4) contains for comparison the pattern obtained with a 7S autoprecipitin of serum III (Cup B). It can be seen that the two lines intersect and that the curvature of the line formed by 7S precipitin and thyroglobulin is quite different from that of the 19S precipitin of serum IV (Cup D) and thyroglobulin. In a recent paper, which appeared while this manuscript was in preparation, Roitt and associates,[15] concluded from the curvature of the precipitin line obtained with Hashimoto's serum and thyroglobulin that the antigen was a large, slowly diffusing molecule. This, of course, is based on the assumption that the antibody was a 7S globulin.

For further confirmation of our data, Hashimoto's sera were subjected to ultracentrifugal fractionation in a partition cell, and both the top and bottom compartments were tested for the presence of autoprecipitins and 19S gamma globulins. Hashimoto's serum I fractions were diluted 1:3. It should be pointed out that the bottom fraction will always contain the active component at a higher concentration than that of the original serum since the bottom compartment was filled with the original serum. From Fig. 5 it can be seen that the contents of the top compartment (Cup A) produce a very strong line with thyroglobulin (center), although only a faint line, which could not be photographed, was formed between anti-19S globulin and traces of 19S globulin

which were still present in the top fraction of serum I. It should be realized that the immunologic test for 19S is very sensitive; this particular antiserum can detect it even when only 5 per cent of the original macroglobulin of the serum is present.

The results obtained with serum II were similar to those described for serum I.

The ultracentrifugal results obtained with serum IV were in agreement with the immunologic data, i.e., hardly any activity was found in the undiluted contents of the top compartment of the partition cell (Fig. 6, Cup F) whereas the contents of the bottom compartment (Cup A) reacted at a dilution of 1:2.

Therefore, from the immunologic and ultracentrifugal data presented, it appears that the autoprecipitins of Hashimoto's sera may be either 7S or 19S gamma globulins. Hashimoto's sera I, II, and III contained the 7S antibody, whereas serum IV contained the 19S precipitin. The examination of many more sera will ultimately decide whether the 7S or 19S globulin is the more common form of this autoprecipitin.

SUMMARY

A new method for the determination of the molecular weight of autoprecipitins is presented. This method depends on the antigenic specificity of the 7S and 19S gamma globulins commonly found in normal sera, and their easy identification with proper antisera in the Ouchterlony double gel diffusion technique.

The examination of Hashimoto's sera containing autoprecipitins against human thyroglobulin by this method has shown that they are either 7S or 19S globulins; these data were confirmed by ultracentrifugal fractionation of 3 of these sera.

Human thyroglobulin is shown to contain two antigenically related components; a third component is antigenically identical with gamma globulin and is probably a contaminant.

We wish to thank Drs. B. Ruebner and R. Benua for sera III and IV. We are also very much indebted to Dr. Mary L. Petermann for her advice and the use of the analytic ultracentrifuge as well as to Mrs. Mary G. Hamilton for fractionating 3 of the sera in the partition cell. The able technical assistance of Mr. Rudolph Smith is acknowledged.

REFERENCES

1. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Ill., 1948, Charles C Thomas.
2. Pressman, D., James, A. W., Yagi, Y., Hiromato, R., Woernley, D., and Maxwell, W. T.: Rapidly Sedimenting Properties of Specifically Precipitating Component of a Hashimoto's Disease Serum, Proc. Soc. Exper. Biol. & Med. **96**: 773, 1957.
3. Korngold, L., and Van Leeuwen, G.: Macroglobulinemia. I. The Antigenic Relationship of Pathological Macroglobulins to Normal γ-Globulins, J. Exper. Med. **106**: 467, 1957.
4. Robbins, J.: Thyroglobulin in Serum After I^{131} Therapy. I. Salting Out, J. Biol. Chem. **208**: 377, 1954.
5. Korngold, L., and Van Leeuwen, G.: Unpublished data.
6. Petermann, M. L., Robbins, J., and Hamilton, M. G.: Sedimentation of the Thyroxine-Binding Protein of Serum in the Partition Cell, J. Biol. Chem. **208**: 369, 1954.

7. Korngold, L., and Lipari, R.: Tissue Antigens of Human Tumors Grown in Rats, Hamsters and Eggs, Cancer Res. **15**: 159, 1955.
8. Korngold, L., and Van Leeuwen, G.: The Effect of the Antigen's Molecular Weight on the Curvature of the Precipitin Line in the Ouchterlony Technic, J. Immunol. **78**: 172, 1957.
9. Korngold, L.: Immunological Cross-Reactions Studied by the Ouchterlony Gel Diffusion Technique—Theory and Practice, J. Immunol. **77**: 119, 1956.
10. Korngold, L., and Van Leeuwen, G.: Formation by One Antigen of Multiple Zones of Precipitate in Double Gel Diffusion: An Immunological Explanation, Federation Proc. **17**: 521, 1958.
11. Korngold, L., and Van Leeuwen, G.: Macroglobulinemia. II. Antisera Specific for Pathological Macroglobulins, J. Exper. Med. **106**: 477, 1957.
12. Doniach, D., and Roitt, G. M.: Auto-Immunity in Hashimoto's Disease and Its Implications, J. Clin. Endocrinol. **17**: 1293, 1957.
13. Wilson, M. W., and Pringle, B. H.: Experimental Studies of the Agar-Plate Precipitin Test of Ouchterlony, J. Immunol. **73**: 232, 1954.
14. Slater, R. J.: The Serum γ-Globulins Defined by Electrophoretic and Immunologic Analyses, Arch. Biochem. **59**: 33, 1955.
15. Roitt, I. M., Campbell, P. N., and Doniach, D.: The Nature of the Thyroid Auto-Antibodies Present in Patients With Hashimoto's Thyroiditis (Lymphadenoid Goitre), Biochem. J. **69**: 248, 1958.

VITAMIN B_{12} ABSORPTION: RELATIONSHIP BETWEEN ORAL ADMINISTRATION AND URINARY EXCRETION OF COBALT^{60}-LABELED CYANOCOBALAMIN FOLLOWING A PARENTERAL DOSE

Study of Doses of 2 to 250 μg in 148 Apparently Healthy Men 20 to 92 Years Old

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ABSORPTION of orally administered radioactive cobalt-labeled vitamin B_{12} (cyanocobalamin or derivative) has been estimated in normal human subjects by difference in the amount of radioactivity administered and that recovered in the feces.[1-5] Absorption has also been correlated with hepatic accumulation[6] and plasma levels[7-10] following oral administration of labeled vitamin B_{12}, and with radioactivity appearing in the urine after oral administration of the labeled vitamin followed by a large parenteral dose of unlabeled material.[11] Correlations between urinary excretion of absorbed vitamin B_{12} and plasma levels[9] and between results from fecal and urinary excretion tests[12] have been demonstrated. The reliability of the urinary excretion technique for distinguishing between normal and markedly impaired absorption of vitamin B_{12}, for smaller oral doses (from 0.1 to 2 μg, usually about 0.5 μg), has been confirmed in many laboratories,[3, 4, 7, 12-16] although abnormally low excretions of radioactive vitamin B_{12} have been noted in terminal cardiorenal disease[7] and in severe renal disease.[17]

The present study was designed, primarily, to evaluate, in healthy subjects, the effect on urinary recovery following a parenteral dose, of varying the oral dose of vitamin B_{12} over a wide range, extending from physiologic or near-physiologic levels to several hundred times these levels. A second consideration has been exploration for possible age differences in absorption for such doses. The dose levels we have selected for study (2 to 250 μg) are higher than those at which an intrinsic factor deficiency, if existent, would seem most likely to be detected, since smaller overlap between results for normal subjects and pernicious anemia patients has been observed when the oral dose was less than 1 μg than when a larger dose was used.[4, 13, 18] Thus, for the higher doses of the present study at least, exploration for age differences constitutes study of agewise variation in efficiency of absorption via mechanisms relatively independent of the amount of intrinsic factor available. While furnishing information on a

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Received for publication Aug. 23, 1957.
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TABLE I. RENAL FUNCTION STUDIES FOR FOURTEEN ELDERLY SUBJECTS IN PRESENT STUDY (VALUES FROM LITERATURE FOR AGEWISE COMPARISON)

ELDERLY SUBJECTS OF PRESENT STUDY										HEALTHY MEN STUDIED BY DAVIES AND SHOCK[19]*					
AGE WHEN RENAL FUNCTION STUDIED (YEARS)	NUMBER OF SUBJECTS	INTERVAL BETWEEN RENAL FUNCTION AND PRESENT STUDIES (YEARS)		INULIN CLEARANCE (ML./MIN./ 1.73 M.²)		DIODRAST OR PAH CLEARANCE (ML./MIN./ 1.73 M.²)		Tm DIODRAST OR PAH (MG. I/MIN./ 1.73 M.²)		INULIN CLEARANCE (ML./MIN./ 1.73 M.²)		DIODRAST CLEARANCE (ML./MIN./ 1.73 M.²)		Tm DIODRAST (MG. I/MIN./ 1.73 M.²)	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
20-29	–									123	16	614	75	54.6	9.5
30-39	–									115	11	649	117	51.0	8.6
40-49	–									121	23	574	112	49.9	9.8
50-59	1	5	–	85.8	–	481†	–	74.7†	–						
		3	–	96.0	–	432	–	60.9	–	99.3	14.6	500	87	45.3	6.3
60-69	1	5	–	81.0	–	342	–	–	–	96.0	25.5	442	80	44.5	9.1
70-79	7	3	1-7	76.6	60.3–90.0	313†	174–427†	66.3†	46.3–75.6						
	1	4	–	69.3	–	242	–	39.2	–	89.0	19.9	354	83	39.1	7.3
80-89	3	3	2-4	55.7	38.6–66.1	266†	155–358†	52.4†	33.5–65.3†	65.3	20.4	289	89	30.8	9.8

*Reported values are rounded off in some cases.
†Indicates PAH (para-aminohippurate).

wide range of doses for which data are meager, our results are also of interest because they represent a large group of healthy subjects studied under conditions in which complete urine collections were facilitated by continuous observation of the subjects during the period of the collections.

MATERIALS AND METHODS

Subjects were 148 men selected from the Gerontology Ward and Chronic Division of the Baltimore City Hospitals (55 to 92 years old) and from a penal institution in the same geographical area (20 to 39 years old). Subjects from the Gerontology Ward and Chronic Division had been there for several years, primarily for indications which were socioeconomic and associated with advanced age rather than illness. Subjects were selected, after review of all available clinical information, as those who were in good general health. All were ambulatory, free of acute or debilitating chronic illness, were in apparently good nutritional status, and believed to be free of hematologic or gastrointestinal disorder. In no subject was there reason for suspicion of diabetes mellitus. Except for the requirement of urinary continence and the general criteria noted above, there was neither an attempt to select subjects with respect to renal function, nor any clinical indication for suspicion of abnormal function. Status of renal function has been evaluated in 14 of the older subjects as a part of other investigations of the Gerontology Branch on renal function in healthy middle-aged and elderly men. Renal function measurements for these subjects* are given in Table I and are seen to be fairly typical for their age groups when compared with the group of healthy subjects studied by Davies and Shock.[19] There was no reason to believe, after review of all available information, that any of the subjects had been administered a source of vitamin B_{12}, except as contained in the institutional diets[20] during the preceding year.

Each subject was given orally (in aqueous solution followed by 2 rinses) 2, 8, 50, or 250 μg of Co^{60}-labeled vitamin B_{12}† (about 2 hours after breakfast and 2 hours before lunch) followed 2 hours after the ingestion of the radioactive vitamin by the intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}.[11] Following the ingestion of the labeled material, urine was collected for either (1) 0 to 12, 12 to 24, and 24 to 48 hours, or (2) 0 to 24 hours only. When collections at 0 to 12 and 12 to 24 hours were made, results were added to obtain a value for 0- to 24-hour excretion of radioactive vitamin. To facilitate complete urine collection, all subjects were under continuous observation during the collection periods. The procedure used for measurement of the radioactivity excreted into the urine was as previously reported[21] except that the aliquot used for measurement in the present studies was half of that collected. That the excreted radioactivity represents "tagged" vitamin B_{12} has been indicated by chromatographic and other analytic studies,[22] and by differences in storage of radioactivity after $Co^{60}Cl_2$ and Co^{60}-vitamin B_{12} administration.[23]

RESULTS

The results, summarized in Fig. 1, show the effect on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying (1) the oral dose of $Co^{60}B_{12}$ in amounts of 2, 8, 50, and 250 μg (Fig. 1, *A*, *B*, *C*, and *D*, respectively) and (2) the urine collection intervals (12, 24, and 48 hours) for each of the age groups and the total group.

*We are indebted to Drs. Nathan W. Shock and David Oursler, of the Gerontology Branch, for making available to us the renal function data on subjects included in the present study.

†For supplies of radioactive and nonradioactive vitamin B_{12}, we are indebted to Dr. Charles Rosenblum and Merck & Company, Inc. The present studies were authorized by the Atomic Energy Commission (Authorization Nos. 34126 and 22808), and we gratefully acknowledge the partial support given in a grant-in-aid from the A.E.C. under contract No. AT(30-1) 1203.

Variations With Age.—The data given in Fig. 1 showed no systematic age difference, for any of the doses or collection periods, in the fraction of dose of cobalt^{60}-vitamin B_{12} excreted in the urine under the experimental conditions of the present study. Further statistical evaluation, in particular for the 24-hour collections when the group aged 20 to 39 years was compared with the group aged 55 to 92 years, revealed no significant age difference: 2 μg, $P > 0.2$; 8 μg, $P > 0.1$; 50 μg, $P > 0.05$; 250 μg, $P > 0.7$. Even when (for 24-hour collections) the group 20 to 39 years old was compared with that 75 years old and older, no consistent age trend was found: 2 μg, $P > 0.1$; 8 μg, $P > 0.01$; 50 μg, $P > 0.6$; 250 μg, $P > 0.2$.

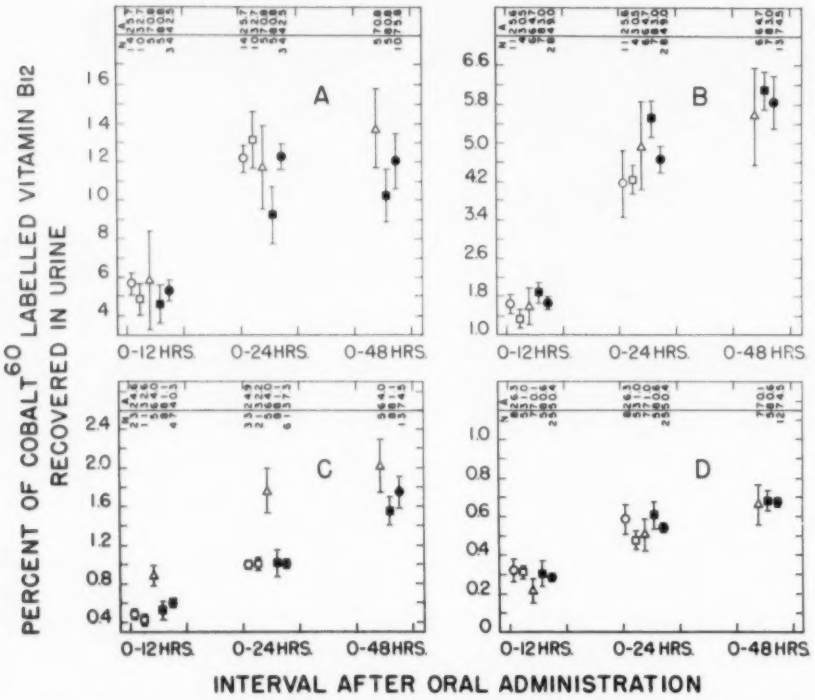

Fig. 1.—Effect, on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying the oral dose for doses of 2, 8, 50, and 250 μg (A, B, C, and D, respectively), and the urine collection interval (12, 24, and 48 hours) for each of the age subgroups and the combined group of healthy men. Key to symbols: ○ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 20 to 29 years; □ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 30 to 39 years; Δ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 55 to 74 years; ■ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 75 to 92 years; ● = mean Co^{60}-vitamin B_{12} recovery for subjects of all ages combined. Vertical lines define limits of the standard error of the mean. At the top of each diagram is given the number of subjects in each age subgroup (N) and the average age for the subjects in the subgroup (A).

Variations With Collection Interval.—For all of the oral doses considered, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the 0- to 12-hour collection. Except for the 2 μg dose, the

amount of vitamin B_{12} appearing in the collection at 24 to 48 hours was one-fifth to one-half as much as that recovered in the first 24 hours after oral administration. For the 2 μg dose, only small amounts of vitamin B_{12} were recovered in the 24- to 48-hour urine collection.

Variations With Oral Dose.—It is apparent from Fig. 1 that with an increasing amount of vitamin B_{12} administered orally, there was a marked decrease in the *fraction* recovered in the urine, under, of course, the conditions of this study. As the oral dose was increased, however, the calculated *amount* of vitamin B_{12} recovered in the urine definitely increased, as shown by the mean values and standard deviations in Table II.

DISCUSSION

A. Effect of Parenteral Dose and Tissue Retention on Results.—The urinary excretion of orally administered vitamin B_{12} in the present study represents only a minimum value for the amount absorbed. The observed difference between the amount apparently absorbed and that excreted in the urine[12] is due, to some extent, to the parenteral dose, since this has been observed to decrease the proportion of an oral dose absorbed from the gastrointestinal tract.[12, 24] The effect of the parenteral dose, however, would seem to be small compared to that due to tissue retention; a large proportion of the absorbed material is retained in the liver.[8, 25] From these considerations and the results of the present study, it would appear that the average amount of vitamin B_{12} absorbed from a single 250 μg dose probably greatly exceeds 1.7 μg, since the latter was the average amount of labeled vitamin B_{12} recovered in the urine within 48 hours after the administration of 250 μg of labeled material.

B. Relation of Amount and Time of Vitamin B_{12} Absorption to Oral Dose.—The character of the relationship obtained in the present study, between oral dose and the amount of vitamin B_{12} excreted in the urine, seems to be in remarkably good agreement with that indicated by the results of Glass and associates.[25] These workers, using the hepatic uptake technique and oral dose range of 0.5 to 50 μg, noted increase in amount and decrease in fraction taken up by the liver in subjects given larger oral doses compared to subjects given smaller doses of labeled vitamin B_{12}. While in agreement with this work, our findings differ from the conclusions of Callender and Evans[1] and Swenseid and co-workers,[26] who used the fecal excretion technique and found optimal absorption at doses not exceeding about 2 and 5 μg, respectively. It seems conceivable that the "plateau" in absorption observed by the latter 2 groups was due to interference associated with successive tests in the same subjects.

The distributions of radioactivity between the several urine collections, in the present study, are consistent with the view that vitamin B_{12} absorption proceeds gradually, and approaches maximal levels sooner for smaller oral doses.

The relationship of the fraction and amount of vitamin B_{12} recovered in the urine to the oral dose of the vitamin are consistent with the views that

TABLE II. AMOUNTS OF VITAMIN B_{12} RECOVERED IN THE URINE FOR VARIOUS ORAL DOSES, FOLLOWED BY A PARENTERAL DOSE, IN APPARENTLY HEALTHY MEN OF AGES 20 TO 92 YEARS

ORAL DOSE (μg)	RECOVERIES OF VITAMIN B_{12} IN URINE (MICROGRAMS)											
	0 TO 12 HOURS				0 TO 24 HOURS				0 TO 48 HOURS			
	N*	RANGE	MEAN	S.D.†	N	RANGE	MEAN	S.D.	N	RANGE	MEAN	S.D.
2	34	0.002-0.28	0.11	0.06	34	0.09-0.48	0.24	0.08	10	0.11-0.41	0.24	0.09
8	28	0.008-0.23	0.13	0.06	28	0.13-0.70	0.37	0.11	13	0.21-0.80	0.46	0.16
50	47	0.06-0.77	0.25	0.14	61	0.33-1.22	0.55	0.17	13	0.45-1.30	0.84	0.30
250	25	0.30-1.65	0.70	0.20	25	0.63-2.53	1.35	0.24	12	0.68-2.80	1.66	0.21

*N = number of subjects.
†S.D. = standard deviation of distribution.

there are at least 2 mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract* in normal people, that these mechanisms act concurrently, and that at different oral dose levels the 2 mechanisms are of relatively different importance. Thus, at low (physiologic) dose levels, the available intrinsic factor is of relatively great importance in influencing the amount absorbed, while at the higher dose levels intrinsic factor is of decreased relative importance and the amount absorbed seems to be more directly related to the amount administered, i.e., a "mass" effect. Although it has not yet been clearly demonstrated to mediate vitamin B_{12} absorption, diffusion would seem to be the simplest mechanism consistent with existing observations of absorption apparently beyond that mediated by intrinsic factor, insofar as these data have been analyzed. It would seem reasonable to suppose that absorption by this mechanism would be negligible or, at least, inadequate to meet nutritional requirements, at low (physiologic) dose levels. At usual dietary levels, then, adequate amounts of the vitamin would be expected to traverse the intestinal mucosa only if adequate amounts of intrinsic factor were available. The manner by which this traversal comes about (at low and high oral dose levels), while the subject of conceptually appealing speculations[25, 27] with which our results are not inconsistent, will remain speculative until many more data have become available and adequately interpreted.

C. Age Differences.—

1. Effect on results of age differences in renal function: In consideration of the possible effect of renal function on the results, it should be noted that subjects included in the present study probably have renal function rather typical for healthy subjects in their respective age groups (see Introduction and Table I). Even for healthy men, however, there is regression of renal function on age.[19] In the absence of such influence in our older subjects, however, the values for excretion of vitamin B_{12} might have been *higher* but not lower than those we observed. Had it happened that significantly less radioactive vitamin B_{12} had been excreted by the older, as compared to the younger, subjects, then the difference might possibly have been explained on the basis of lower renal function in the older subjects. Such did not occur, however, and the fact that the urine collections for the older men contained as much radioactivity as did those for the young subjects cannot be explained on the basis of age differences in renal function.

Failure to observe decreased excretion of Co^{60}-vitamin B_{12} in the older subjects we have studied was not likely to have been due to decreased tissue retention of absorbed vitamin B_{12}, in the old as compared to the young men, in view of the following considerations: (1) amounts of radioactive vitamin B_{12} retained by the liver after oral administration were found by Glass and coworkers[6] to be not significantly different in old and young men; (2) the large parenteral dose of vitamin B_{12}, given to all subjects, would be expected to

*Since this paper was submitted, there has appeared a paper by Doscherholmen and Hagen which supports the existence of a dual mechanism in the absorption of vitamin B_{12}. (Doscherholmen, A., and Hagen, P. S.: A Dual Mechanism of Vitamin B_{12} Plasma Absorption, J. Clin. Invest. 36: 1551-1557, 1957.)

give a higher degree of tissue saturation with the vitamin (before the radioactive vitamin entered the plasma) than would otherwise have been the case; (3) retention of parenterally administered doses of 20 to 75 μg of vitamin B_{12} is probably even greater in old men than in young men.[28]

2. Comparison of our results with those of other studies related to absorption of vitamin B_{12} and aging: Chow and associates[21] measured the urinary excretion of radioactive vitamin B_{12} following oral administration of 2 μg of labeled material and a large parenteral dose; in 40 healthy subjects 19 to 85 years old, no significant age difference was observed. Using the hepatic uptake method and a test dose of 0.5 μg of vitamin B_{12}, Glass and co-workers[6] found no significant age difference in radioactive vitamin B_{12} uptake by the liver in 60 subjects aged 18 to 90 years who were normal or had some irrelevant disorder. In other reports, the number of "healthy" or "control" subjects has been inadequate for statistical treatment of the data for agewise study (8 "controls" in Schilling's[29] paper, for example) or adequate information on ages of subjects has been omitted.[12]

3. Relation of the present study to evidence for age differences in serum vitamin B_{12} levels: Recent studies in healthy human subjects in our laboratories[20] and elsewhere[30, 31] have indicated that there is a decrease in serum concentration of vitamin B_{12} microbiologic activity with advancing age. The results of the present study for doses of 2 to 250 μg, other studies[21] for the 2 μg dose, and studies of hepatic uptake for the 0.5 μg dose[6] do not support the contention that age differences in serum levels of vitamin B_{12} are due to impairment of absorption of the vitamin. It is possible, although not yet clearly demonstrated, that the lower levels of serum vitamin B_{12} observed in the aged subjects may have been associated with decreased ingestion of vitamin B_{12} due to dietary selection by the subjects themselves,[20] in the absence of any significant impairment in vitamin B_{12} absorption. It should be emphasized, however, that tests using oral doses of 2 μg and larger, such as in the present study, are not representative of absorption for the smaller doses in which the best separations have been found between patients with intrinsic factor deficiency and normal subjects.[4, 13, 18] Smaller doses, moreover, would seem to closely approach levels more frequently found in the diet (and are, in this sense, physiologic doses). The data do suggest, however, that amounts of vitamin B_{12} absorbed from oral doses in the 2 to 250 μg range are not significantly less in old men than in young men.

SUMMARY

1. Labeled material appearing in the urine in 12, 24, and 48 hours after oral administration of 2, 8, 50, or 250 μg of cobalt^{60}-labeled vitamin B_{12} (cyanocobalamin or derivative), followed after 2 hours by intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}, has been estimated in 148 apparently healthy men 20 to 92 years old.

2. With increasing oral dose, for the range considered, there was observed marked decrease in *fraction* but considerable increase in *amount* of vitamin

B_{12} appearing in the urine. For all doses, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the collection at 0 to 12 hours. Except for the 12 μg dose, the amount of vitamin B_{12} appearing in the 24- to 48-hour collection was one-fifth to one-half as much as that recovered in the first 24 hours after oral administration. Mean recoveries in micrograms of labeled vitamin B_{12} in the urine ± standard deviation of distribution for each of the oral doses and for 0 to 12, 0 to 24, and 0 to 48 hours after oral administration, respectively, were: (1) *2 μg*: 0.11 ± 0.06, 0.24 ± 0.08, 0.24 ± 0.09; (2) *8 μg*: 0.13 ± 0.06, 0.37 ± 0.11, 0.46 ± 0.16; (3) *50 μg*: 0.25 ± 0.14, 0.55 ± 0.17, 0.84 ± 0.31; (4) *250 μg*: 0.70 ± 0.20, 1.35 ± 0.24, 1.66 ± 0.21.

3. The data are consistent with the views that there are at least two mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract, that these mechanisms act concurrently and have relatively different importance at different dose levels, and that absorption proceeds gradually, approaching maximal levels sooner for smaller dose levels.

4. Age differences in urinary excretion or orally administered material were not demonstrated for any of the doses under the experimental conditions of the study. The oral doses used, however, were higher than those for which best separation has been demonstrated between normal subjects and patients with intrinsic factor deficiency. The excreted material probably represents that absorbed, to some extent for the smallest dose and in large proportion for the larger doses, by a mechanism relatively independent of the amount of intrinsic factor available. The efficiency of such a mechanism, insofar as indicated by the urinary excretion test, was not impaired in the older, as compared to the younger, subjects.

We wish to express our gratitude to many individuals of the staffs of the Gerontology Branch, Baltimore City Hospitals and the Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health, for their aid in the present study, including technical assistance and reading of the manuscript; to the Nursing Staff of the Baltimore City Hospitals for observation of subjects during the periods of urine collections; and to Drs. Patricia McIntyre and Julius Krevans for their interest in the work.

REFERENCES

1. Callender, S. T., and Evans, J. R.: Observations on the Relationship of Intrinsic Factor to the Absorption of Labelled Vitamin B_{12} From the Intestine, Clin. Sc. **14**: 387-393, 1955.
2. Halsted, J. A., Gasster, M., and Drenick, E. J.: Absorption of Radioactive Vitamin B_{12} After Total Gastrectomy, New England J. Med. **251**: 161-168, 1954.
3. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H.: Studies of Excretion (and Absorption) of Co^{60} Labeled Vitamin B_{12} in Pernicious Anemia, Tr. A. Am. Physicians **65**: 214-222, 1952.
4. Krevans, J. R., Conley, C. L., and Sachs, M.: Radioactive Tracer Tests for the Recognition and Identification of Vitamin B_{12} Deficiency States, J. Chron. Dis. **3**: 234-252, 1956.
5. Meyer, L. M.: Oral Administration of Co^{60} Vitamin B_{12} in Pernicious Anemia, Proc. Soc. Exper. Biol. & Med. **82**: 490-491, 1953.
6. Glass, G. B. J., Goldbloom, A. A., Boyd, L. J., Laughton, R., Rosen, S., and Rich, M.: Intestinal Absorption and Hepatic Uptake of Radioactive Vitamin B_{12} in Various Age Groups and the Effect of Intrinsic Factor Preparations, Am. J. Clin. Nutrition **4**: 124-133, 1956.
7. Goldberg, S. R., Trivedi, B. K., and Oliner, L.: Radioactive Vitamin B_{12} Studies. Experience With the Urinary Excretion Test and the Measurement of Absorbed Plasma Radioactivity, J. Lab. & Clin. Med. **49**: 583-589, 1957.

8. Booth, C. C., and Mollin, D. L.: Plasma, Tissue and Urinary Radioactivity After Oral Administration of Co^{56} Labelled Vitamin B_{12}, Brit. J. Haemat. **2:** 223-236, 1956.
9. Doscherholmen, A., and Hagen, P. S.: Radioactive Vitamin B_{12} Absorption Studies: Results of Direct Measurement of Radioactivity in the Blood, Blood **12:** 336-346, 1957.
10. Doscherholmen, A., and Hagen, P. S.: A Dual Vitamin B_{12} Absorption Mechanism, J. Clin. Invest. **36:** 884, 1957.
11. Schilling, R. F.: Intrinsic Factor Studies. II. The Effect of Gastric Juice on the Urinary Excretion of Radioactivity After the Oral Administration of Radioactive Vitamin B_{12}, J. LAB. & CLIN. MED. **42:** 860-866, 1953.
12. Callender, S. T., and Evans, J. R.: The Urinary Excretion of Labelled Vitamin B_{12}, Clin. Sc. **14:** 295-302, 1955.
13. Meyer, L. M., Becera-Garcia, A., Goldman, A., and Stern, P. A.: Oral Administration of Co^{60} Vitamin B_{12} to Normal Persons, Patients With Pernicious Anemia, and Subjects With Various Medical Disorders, J. Appl. Physiol. **6:** 263-268, 1953.
14. Swenseid, M. E., Halsted, J. A., and Libby, R. L.: Excretion of $Cobalt^{60}$-labeled Vitamin B_{12} After Total Gastrectomy, Proc. Soc. Exper. Biol. & Med. **83:** 226-228, 1953.
15. Callender, S. T., Turnbull, A., and Wakisaka, G.: Estimation of Intrinsic Factor of Castle by Use of Radioactive Vitamin B_{12}, Brit. M. J. **1:** 10-13, 1954.
16. Halsted, J. A., Swenseid, M. E., Gasster, M., and Lewis, P. M.: Effect of Antibiotics on Intestinal Absorption of Vitamin B_{12} in Disorders of the Gastrointestinal Tract, Am. J. Med. **19:** 297-298, 1955.
17. Rath, C. E., McCurdy, P. R., Duffy, B. J., Jr., and Howley, J. R.: Value and Limitations of $Cobalt^{60}$ B_{12} Test, Am. J. Med. **20:** 954-955, 1956.
18. Meyer, L. M., Jiminez-Casada, M., and Arkun, S. N.: Further Observations on the Oral Administration of Co^{60} Vitamin B_{12} to Normal Persons, Patients With Pernicious Anemia, and Subjects With Various Medical Disorders, Acta med. scandinav. **153:** 153-158, 1955.
19. Davies, D. F., and Shock, N. W.: Age Changes in Glomerular Filtration Rate, Effective Renal Plasma Flow and Tubular Excretory Capacity in Adult Males, J. Clin. Invest. **29:** 496-507, 1950.
20. Gaffney, G. W., Horonick, A., Okuda, K., Meier, P., Chow, B. F., and Shock, N. W.: Vitamin B_{12} Serum Concentrations in 528 Apparently Healthy Human Subjects of Ages 12-94, J. Gerontol. **12:** 32-38, 1957.
21. Chow, B. F., Gilbert, J. P., Okuda, K., and Rosenblum, C.: The Urinary Excretion Test for Absorption of Vitamin B_{12}. I. Reproducibility of Results and Agewise Variation, Am. J. Clin. Nutrition **4:** 142-146, 1956.
22. MacLean, L. D., and Bloch, H. S.: Gastrointestinal Absorption and Urinary Excretion of Vitamin B_{12}-Co^{60}, Proc. Soc. Exper. Biol. & Med. **87:** 171-174, 1954.
23. Meyer, L. M., Berlin, N., Jiminez-Casada, M., and Arkun, S. N.: Vit. B_{12} Distribution, Determined by Surface Body Counting Following Parenteral Administration of Co^{60} Vit. B_{12}, Proc. Soc. Exper. Biol. & Med. **91:** 129-131, 1956.
24. Baker, S. J., and Mollin, D. L.: The Relationship Between Intrinsic Factor and the Intestinal Absorption of Vitamin B_{12}, Brit. J. Haemat. **1:** 46-51, 1955.
25. Glass, G. B. J., Boyd, L. J., and Stephanson, L.: Intestinal Absorption of Vitamin B_{12} in Humans as Studied by Isotope Technic, Proc. Soc. Exper. Biol. & Med. **86:** 522-526, 1954.
26. Swenseid, M. E., Gasster, M., and Halsted, J. A.: Limits of Absorption of Orally Administered Vitamin B_{12}: Effect of Intrinsic Factor Sources, Proc. Soc. Exper. Biol. & Med. **86:** 834-836, 1954.
27. Conley, C. L.: in discussion of paper by Chow, B. F.: The Role of Vitamin B_{12} in Metabolism, South. M. J. **45:** 604-612, 1952.
28. Watkin, D. W., Lang, C. A., Chow, B. F., and Shock, N. W.: Agewise Differences in the Urinary Excretion of Vitamin B_{12} Following Intramuscular Administration, J. Nutrition **50:** 341-349, 1953.
29. Schilling, R. F.: The Absorption and Utilization of Vitamin B_{12}, Am. J. Clin. Nutrition **3:** 45-51, 1955.
30. Boger, W. P., Wright, L. D., Strickland, S. C., Gylfe, J. S., and Ciminera, J. L.: Vitamin B_{12}: Correlation of Serum Concentrations and Age, Proc. Soc. Exper. Biol. & Med. **89:** 375-378, 1955.
31. Mollin, D. L., and Ross, G. I. M.: The Vitamin B_{12} Concentrations of Serum and Urine of Normals and Patients With Megaloblastic Anemias and Other Diseases, J. Clin. Path. **5:** 129-139, 1952.

A SEROLOGIC AND ELECTROPHORETIC STUDY OF THE MALIGNANT AND PROLIFERATIVE DISORDERS OF THE HEMATOPOIETIC AND RETICULOENDOTHELIAL SYSTEMS

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THE distribution and concentration of various plasma protein constituents are altered in many disorders of the hematopoietic and reticuloendothelial systems. Such changes have been demonstrated by electrophoretic[26] and serologic techniques[27] in patients with lymphoma, leukemia, and multiple myeloma.

The purpose of this paper is to study the serum protein changes in patients with malignant hematopoietic disorders by the use of electrophoretic and serologic techniques and to relate these variations to the type of disease, the clinical status of the patients, and the effect of therapeutic agents employed in the treatment of these disorders.

MATERIALS AND METHODS

The subjects of this study were 99 patients admitted to the hematology clinic or wards of the First and Third Medical Services (Tufts), Boston City Hospital, between September, 1957, and February, 1958, in whom the diagnosis of malignant lymphoma, leukemia, myeloproliferative disorder, or multiple myeloma was confirmed by peripheral blood studies and bone marrow aspiration and/or tissue biopsies. Healthy adults, both men and women, 20 to 45 years of age, served as controls.

Serum protein filter paper electrophoresis was performed according to a modification of the methods of Williams and associates[28] and Jencks and co-workers,[29] using a Spinco Model R duostat or Heathkit duostat as the power source, a Spinco Model R paper electrophoresis cell, Spinco filter paper wicks and strips, and barbital buffer, pH 8.6, ionic strength 0.075, and 0.1 M. Electrophoresis of the sera was performed at 250 volts over a 6-hour period. The strips were dried in an oven at 45° C., stained with bromphenol

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This project was supported in part by a grant from The Atomic Energy Commission, AT (30-1) 1871, and in part by Research Grant C-3171 from the National Cancer Institute, United States Public Health Service.

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Received for publication Aug. 7, 1958.

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blue, washed in 0.5 per cent acetic acid (v/v) for 10 minutes, and fixed for 30 minutes in a solution of 0.5 per cent sodium acetate in 2 per cent acetic acid (w/v). The strips were dried in an oven at 45° C. once more, and scanned on Spinco Analytrol charts, utilizing a Spinco Model RB Analytrol to determine the percentage contribution of each protein fraction. Because of the nonlinear relationship between dye concentration and dye uptake,[30] and the nonlinear relationship between dye concentration on paper and scanner response,[31] a correction factor for the albumin had to be applied. This was determined by eluting the desired segments of the strips from the sera in 0.01 N sodium hydroxide as outlined by Crook and associates.[31] Under our conditions, this correction factor for the albumin was 1.5.

Total protein was determined by the biuret method[32] using a commercial standard protein solution* as a control.

The blood groups of the normal controls and patients were determined by using both known cells and commercial antisera.† Isoagglutinin titers were determined by the serial dilution of 0.1 ml. of serum with equal volumes of normal saline in serologic tubes. Fresh known group A and/or B cells, depending on the subject's blood type, in the amount of 0.1 ml., were added to each tube, allowed to stand for one hour at room temperature, and centrifuged at 1,500 r.p.m. for 2 minutes. The tubes were then shaken gently, and checked for macroscopic agglutination. The greatest dilution in which agglutination of the red cells occurred was taken as the end point and reported as the titer. It became apparent early in the study that many titers were undetectable at the 1:2 level of dilution. An initial tube, consisting of undiluted serum, was used thereafter, and the other dilutions carried out as described.

RESULTS

In the interpretation of the data to be presented, 3 points should be kept in mind. First, the concentration of each serum protein fraction is expressed in terms of per cent of the total protein rather than as grams per cent of plasma. Because results are expressed in this manner, any change in the concentration of one fraction will lead to complementary changes in the percentage concentration of the other fractions. Second, the electrophoretic data shown in the various tables represent uncorrected values for all constituents. This was done to avoid the inaccuracy involved in using a correction factor for abnormal globulins whose affinity for dye is unpredictable. Third, in the compilation of the isoagglutinin titers, the anti-A titers from patients and controls of groups O and B were considered as one group. Similarly, the anti-B titers from patients and controls of groups O and A were grouped together. Since there was no change in isoagglutinin titers with treatment in any of the diseases studied, and there was no difference between the titers of the patients in remission and those of the patients in relapse, the titers of all patients in each disease were considered graphically as one group.

In every disease state the observations on patients in relapse who were being treated and the observations on patients in remission who were untreated were compared with the controls and with each other. The significance of the comparisons was measured by the "t" test.[33] Significant results are specifically noted, and the direction of change is indicated.

*Pro-Sol, obtained from Standard Scientific Corporation, Flushing, N. Y.
†Obtained from Dade Reagents, Incorporated, Miami, Fla.

TABLE I. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN NORMAL SUBJECTS

VALUES	NUMBER OF SUBJECTS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Mean	39	47.0	6.0	13.0	15.0	20.0	6.4
Standard deviation		4.7	1.7	2.7	2.0	3.5	0.6
Normal range: Mean ±2 standard deviations		37-56	3-9	9-18	11-19	13-27	5.1-7.7

Normal Values.—Table I shows the normal values for the electrophoretic fractionation of serum proteins and total protein concentration in 39 control subjects.

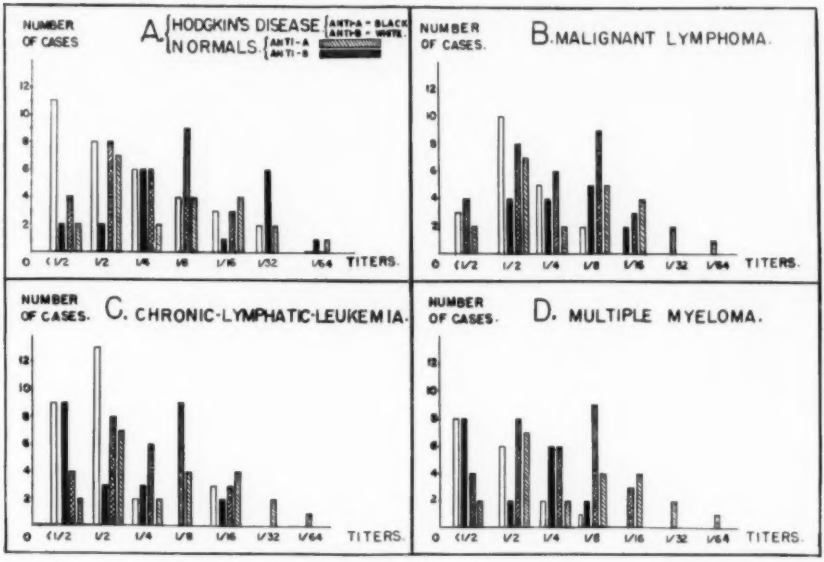

Fig. 1.—The distribution of isoagglutinin titers in normal subjects is contrasted with the distribution of isoagglutinin titers in patients with (A) Hodgkin's disease, (B) malignant lymphoma, (C) chronic lymphatic leukemia, and (D) multiple myeloma.

Fig. 1 shows the distribution of isoagglutinin titers in 36 of these normal subjects. Among the control group, 13 subjects were group A, 5 group B, 1 group AB, and 17 group O. The anti-A titers were considerably greater than the anti-B titers, averaging 1:11.3 as compared with an average anti-B titer of 1:5.5. Repeated tests on two normal individuals over a 6-week period revealed that the isoagglutinin titers maintained a relatively constant level.

Hodgkin's Disease.—The electrophoretic and statistical data for the sera from 20 patients with Hodgkin's disease are shown in Table II. The group

TABLE II. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH HODGKIN'S DISEASE

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	9	18	39.3	8.1	15.2	16.9	21.6	6.0
	Standard deviation			7.9	2.8	3.4	3.5	6.6	0.9
Group 2 (patients in remission)	Mean	11	27	46.3	7.2	13.7	16.1	16.9	6.4
	Standard deviation			10.4	2.1	4.8	5.4	5.9	1.0
Patients in relapse compared with controls	Significance (p)			0.001↓*	0.007↑*	0.02↑	0.001↑	0.4	0.06
Patients in remission compared with controls	Significance (p)			0.70	0.01 ↑	0.5	0.3	0.008↓	0.1
Patients in relapse compared with patients in remission	Significance (p)			0.01 ↓	0.2	0.2	0.6	0.02 ↑	0.001↓

*In this and the following tables, ↓ refers to significantly decreased values and ↑ refers to significantly increased values.

consisted of cases of Hodgkin's paragranuloma, Hodgkin's granuloma, and Hodgkin's sarcoma. Nine cases in relapse received varying forms of therapy during the course of study, including prednisone* (2 cases), nitrogen mustard (2 cases), and x-irradiation and nitrogen mustard (5 cases).

In the sera of patients in relapse, the albumin levels were significantly decreased below normal, while the alpha-1, alpha-2, and beta globulin fractions were significantly elevated. The sera from 11 patients in remission exhibited two significant deviations: an increase in alpha-1 globulin values and a decrease in gamma globulin levels. The albumin and total protein levels of the sera from patients in relapse were significantly lower, and the gamma globulin levels were significantly higher than the corresponding values in the sera from the patients in remission.

The distribution of isoagglutinin titers for the patients with Hodgkin's disease is shown in Fig. 1, A. There was no difference between the patients and controls. There was no correlation between gamma globulin concentration and isoagglutinin titers.

Malignant Lymphoma.—This group consisted of 19 cases of lymphocytoma, lymphoblastoma, lymphosarcoma, and reticulum cell sarcoma. Nine patients were in remission. Ten patients were in relapse and received therapy during the period of study, including nitrogen mustard (5 cases), x-irradiation (1 case), prednisone (2 cases), prednisone and nitrogen mustard (1 case), and x-irradiation and nitrogen mustard (1 case).

The average alpha-2 and gamma globulin levels of the sera from the patients in relapse were significantly less than the corresponding normal values. The average gamma globulin values of the sera from the patients in remission was significantly decreased below normal levels. There was no significant statistical difference between the serum protein distribution and concentration of the patients in relapse and of those in remission.

The distribution of the isoagglutinin titers in patients with malignant lymphoma is shown in Fig. 1, B. The lower titers predominated. There was no correlation between isoagglutinin titers and gamma globulin levels.

One patient with severe hypogammaglobulinemia had consistent anti-B titers of 1:2. At this time, the patient had pneumonia which persisted for 6 weeks. Following this episode, the gamma globulin rose to a normal level, although the titers did not change.

One patient was excluded from the statistical analysis. This patient (R. S.), a 60-year-old white man with lymphocytoma, exhibited a homogeneous serum component in the gamma region (Fig. 2). No titers were performed in this case.

Chronic Lymphocytic Leukemia.—The sera from 13 patients with chronic lymphocytic leukemia were studied. Four patients were in remission. Nine

*The prednisone (Meticorten) used in this study was kindly supplied by the Schering Corporation, Bloomfield, N. J.

patients were in relapse and received varying therapy including CB1348* (2 cases), x-ray (1 case), prednisone (2 cases), and chlorambucil and nitrogen mustard (1 case).

In relapse, there were significantly increased alpha-1 globulin levels and significantly decreased gamma globulin values, while in remission there was a significant decrease in the gamma globulin fraction alone. Comparison of the sera from the patients in relapse and those in remission revealed no significant differences between the two groups.

Six patients had gamma globulin levels of less than 5 per cent. Five of these patients suffered severe respiratory infections during the course of the study. One of these patients had both pneumonia and pyelonephritis. In this latter case, the gamma globulin returned to normal levels following the subsidence of the infection.

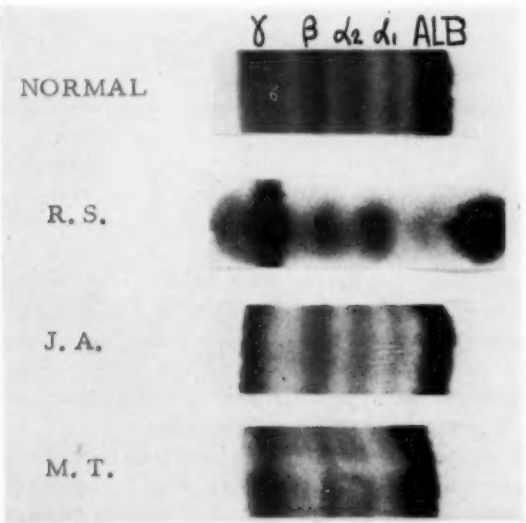

Fig. 2.—Paper electrophoretic patterns of the serum proteins of 3 patients with lymphocytoma (R. S.), chronic lymphatic leukemia (J. A.), and polycythemia vera (M. T.), respectively, showing an abnormal gamma component, which is frequently observed in multiple myeloma.

The isoagglutinin titers in patients with chronic lymphocytic leukemia are graphically shown in Fig. 1, C. The titers are considerably lower than those of the normal subjects. Although this group showed both hypogammaglobulinemia and decreased isoagglutinin titers, there was no correlation between these two factors in the individual case.

One patient was excluded from the statistical analysis. This patient (J. A.), a 60-year-old white man, had been diagnosed as chronic lymphocytic leukemia 5 years previously. He had a persistent sharp myeloma-like peak in

*The chlorambucil used in this study was kindly furnished as p-di(2-chloroethyl) aminophenylbutyric acid by Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

the gamma region (Fig. 2). There was no associated proteinuria. Cryoglobulins were not present. He had recurrent bouts of bronchitis and one episode of pneumonia. Terminally, he developed oral moniliasis. His anti-A and anti-B titers were 1:1 on several occasions. Postmortem examination revealed widespread visceral involvement by leukemic infiltrates. Sections were stained with phloxine-methylene blue and methyl green-pyronine Y (Pappenheim-Saathof method).[34] No plasma cells were noted. The leukemic cells showed no affinity for the pyronine.

Chronic Myelogenous Leukemia.—The sera from 13 patients with chronic myelogenous leukemia were studied. Twelve patients were in relapse and received therapy during the course of study. Ten patients were treated with Myleran* and two cases received prednisone.

The electrophoretic and statistical data are presented in Table III. In relapse, the alpha-2 and gamma globulin levels of the sera were significantly decreased, while there was a significant increase in the beta globulin fraction. Repeated observation of the serum from the patient in remission revealed no significant abnormalities. When the sera from the patients in relapse were compared with that of the patient in remission, only one significant difference, a decrease in the alpha-2 globulin fraction in the former group, was noted.

The isoagglutinin titers in chronic myelogenous leukemia followed a normal distribution.

Myeloid Metaplasia.—Five patients with myeloid metaplasia were studied. Four patients were in remission. One patient received local x-irradiation during the period of study.

The electrophoretic and statistical data are presented in Table IV. The serum of the patient in relapse was significantly decreased in its alpha-2 and beta globulin levels, and significantly increased in its gamma globulin level on repeated observation. The 4 patients in remission had decreased serum alpha-2 and gamma globulin values. When the serum of the patient in relapse was compared with that of the patients in remission, there was a significant decrease in the beta globulin fraction and a significant increase in the gamma globulin level.

The isoagglutinin titers in myeloid metaplasia were low, but the number of determinations was insufficient to render definite conclusions.

Polycythemia Rubra Vera.—The sera from 4 patients in this group were studied. There was no significant difference between the control sera and patient sera. The isoagglutinin titers followed the normal distribution.

One patient (M. T.), a 52-year-old Negro woman, was excluded from the statistical analysis because she had a sharp persistent myeloma-like peak in the gamma region (Fig. 2). Polycythemia had been diagnosed in 1954. Liver function tests were normal. There was no proteinuria. Cryoglobulins were not present. Bone marrow aspirations on 3 recent occasions revealed hypocellular marrow with a shift to the left. There was no increase in plasma cells. The anti-A and anti-B titers were 1:32 and 1:16, respectively, on several occasions.

*Busulfan (1,4-dimethanesulfonyloxybutane).

TABLE III. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 patients in relapse	Mean	12	36	47.2	5.8	11.7	16.7	18.7	6.2
	Standard deviation			10.8	1.9	2.2	4.2	2.0	0.8
Group 2 patients in remission	Mean	1	3	43.0	5.3	13.4	17.1	20.5	5.74
	Standard deviation			7.7	0.6	0.8	2.9	5.9	1.45
Patients in relapse compared with controls	Significance (p)			0.9	1.0	0.02 ↓*	0.001↑	0.05↓	0.40
Patients in remission compared with controls	Significance (p)			0.4	0.1	0.5	0.2	0.9	0.4
Patients in relapse compared with patients in remission	Significance (p)			0.4	0.2	0.005↓	0.9	0.5	0.5

*See footnote to Table II.

TABLE IV. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH MYELOID METAPLASIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	1	4	48.3	5.4	7.8	11.6	24.3	6.3
	Standard deviation			6.29	1.8	2.1	0.8	1.6	0
Group 2 (patients in remission)	Mean	4	10	51.9	6.6	8.2	15.5	15.5	6.4
	Standard deviation			9.9	1.9	2.8	4.0	4.2	0.7
Patients in relapse compared with controls	Significance (p)			0.7	0.6	0.001↓*	0.001↓	0.001↑	0.3
Patients in remission compared with controls	Significance (p)			0.1	0.3	0.001↓	0.6	0.003↓	0.9
Patients in relapse compared with patients in remission	Significance (p)			0.4	0.3	0.8	0.003↓	0.001↑	0

*See footnote to Table II.

TABLE V. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH ACUTE LEUKEMIA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	4	7	35.1	7.5	14.6	13.1	29.3	5.8
	Standard deviation			10.2	1.0	3.3	2.1	8.6	0.7
Group 2 (patients in remission)	Mean	6	17	52.8	5.5	10.7	14.4	16.9	6.8
	Standard deviation			9.5	1.8	3.3	3.9	5.2	1.0
Patients in relapse compared with controls	Significance (p)			0.02 ↓*	0.005↑	0.3	0.08	0.02 ↑	0.01
Patients in remission compared with controls	Significance (p)			0.02 ↑	0.3	0.01↓	0.5	0.03 ↓	0.4
Patients in relapse compared with patients in remission	Significance (p)			0.001↓	0.001↑	0.02↓	0.3	0.005↑	0.06

*See footnote to Table II.

Acute Leukemia.—The sera from 10 patients with acute leukemia were studied, including acute lymphocytic leukemia (6 cases), acute monocytic leukemia (3 cases), and histiocytic leukemia (1 case). Four patients were treated with prednisone, and two patients received Meticorten and 6-mercaptopurine.

The electrophoretic and statistical data are presented in Table V. In relapse, there were significantly decreased serum albumin and total protein levels and significantly increased alpha-1 and gamma globulin values. In remission, there were significantly elevated serum albumin values and significantly decreased values of alpha-2 and gamma globulin. When the sera from the patients in relapse were compared with that of the patients in remission, the albumin and alpha-2 globulin fractions were significantly lower, and the alpha-1 and gamma globulin fractions were significantly higher in the former group.

There was no difference between the isoagglutinin titers of the 10 patients with acute leukemia and those of the control group. Moreover, there was no correlation between gamma globulin levels and isoagglutinin titers in this group.

Multiple Myeloma.—Twelve patients with multiple myeloma were studied, including one case of plasma cell leukemia. Eight patients received therapy including urethane (6 cases), x-ray (1 case), and prednisone (1 case). Ten patients had homogeneous serum constituents with the following mobilities: 4 cases, fast gamma; 3 cases, intermediate gamma; 2 cases, slow gamma; and 1 case, slow and fast gamma.

The electrophoretic and statistical data for this group are presented in Table VI. The 4 patients in relapse had significantly decreased serum levels of albumin and significantly elevated values for the alpha-1 and gamma globulin fractions. The 8 patients in remission had significantly low serum albumin and beta globulin values and significantly elevated values for the gamma globulin and total protein concentration. When the sera from the patients in relapse were compared with that of the patients in remission, the values for the albumin fraction and total protein concentration were significantly lower, and the levels of the alpha-1 and gamma globulin fractions were significantly higher in the former group.

In 3 cases, treatment was of such short duration that the effects could not be evaluated. X-irradiation to localized lesions and Meticortin produced no effect on the protein distribution. There was a decrease in the height of the peak in 4 cases treated with urethane. One patient had a decreased peak initially under therapy which subsequently increased. In another (M. C.), the band disappeared completely from the tracings of a sample taken 25 days after urethane therapy. Technical and clerical errors appeared to be satisfactorily ruled out as an explanation for this striking change. Electrophoresis two months later revealed reappearance of the band (Fig. 3, C). Unfortunately, intermediate sera were not available. Urethane therapy produced a considerable decrease in the alpha-1, beta, and gamma globulins, and

TABLE VI. SERUM PROTEIN DISTRIBUTION AND CONCENTRATION IN PATIENTS WITH MULTIPLE MYELOMA

	VALUES	NUMBER OF PATIENTS	NUMBER OF OBSERVATIONS	ALBUMIN (%)	ALPHA-1 GLOBULIN (%)	ALPHA-2 GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	TOTAL PROTEIN (GM. %)
Group 1 (patients in relapse)	Mean	4	12	30.6	9.3	12.1	12.7	35.7	6.2
	Standard deviation			7.3	2.7	3.1	3.4	6.1	0.4
Group 2 (patients in remission)	Mean	8	19	42.6	5.8	12.0	12.3	25.8	7.3
	Standard deviation			7.2	2.1	5.3	6.5	8.4	1.7
Patients in relapse compared with controls	Significance (p)			0.001↓*	0.005↑	0.5	0.1	0.001↑	0.5
Patients in remission compared with controls	Significance (p)			0.01 ↓	0.7	0.4	0.04↓	0.003↑	0.03↑
Patients in relapse compared with patients in remission	Significance (p)			0.001↓	0.005↑	1.0	0.8	0.005↑	0.03↓

*See footnote to Table II.

a significant elevation of the albumin fraction in 3 of 6 cases. There was a concurrent decrease in the total protein concentrations. With continued urethane therapy in 2 of these 3 cases, the albumin once more decreased and was accompanied by a rise in the alpha-1, beta, and gamma globulins, and total protein concentration.

The distribution of the isoagglutinin titers is shown in Fig. 1, D. There was a striking decrease in the titers in multiple myeloma. Correlation between the presence or absence of abnormal protein components with isoagglutinin titers could not be determined because of the limited number of cases without these proteins.

Four patients had pneumonia during the period of study, while one patient succumbed to a staphylococcal septicemia which arose in a buttock abscess.

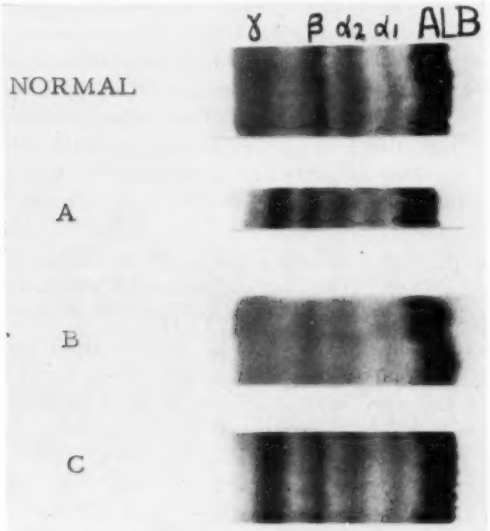

Fig. 3.—Paper electrophoretic patterns of the serum proteins of a patient (M. C.) with multiple myeloma, exhibiting (A) an intermediate gamma band prior to urethane therapy, (B) disappearance of the myeloma protein with urethane therapy, and (C) the reappearance of the abnormal component on continued therapy.

DISCUSSION

Our results are in general agreement with those of other investigators. Abnormalities are frequently present in the serum of patients with diseases of the reticuloendothelial system, the gamma globulins being most frequently altered.

It is interesting to note that the findings in Hodgkin's disease differ in several respects from other types of malignant lymphoma and chronic lymphatic leukemia. First, hypoalbuminemia is a significant finding in the patients with Hodgkin's disease in relapse, while this is not so in the other

groups studied. Although this may simply be a nonspecific response to the severity of the illness, the case material does not give support to this theory. Second, there was an increase in the alpha and beta globulins with a normal gamma value in this same group of patients, while those with lymphoma and lymphatic leukemia had variable changes in alpha globulins associated with a significant decrease in gamma globulins. Third, the isoagglutinins were present in normal titer, while those in the lymphoma-lymphatic leukemia group were low.

These findings differ from some of the reported studies of electrophoresis in Hodgkin's disease, which have frequently revealed an increase in gamma globulin.[1-3, 7, 35] Also, in contrast to other reports of serum electrophoresis in cases of lymphoma which have described an increase in alpha, beta, and gamma globulins, a significant decrease in gamma globulin was demonstrated in the present series. Our observations of hypogammaglobulinemia in lymphatic leukemia, however, do confirm observations of others.[2, 4, 6, 9] The discrepancy between our results and those of other investigators may be partially explained by the difference in separation of protein fractions in the Tiselius apparatus and by the clinical status of the patients themselves.

Studies of isoagglutinins in the lymphoma-lymphatic leukemia group revealed a definite decrease in titer, and although there was no direct correlation with total gamma globulin, this finding was coincident with the tendency toward hypogammaglobulinemia.

In chronic myelogenous leukemia, serum protein changes were also found. Both alpha-2 and gamma globulins were significantly decreased, and beta globulin increased, in agreement with other reports.[1, 2, 4, 6, 9]

The other myeloproliferative diseases studied varied somewhat from the leukemic group. The alpha-2 and beta globulins were significantly decreased, and gamma globulins were normal or low in myeloid metaplasia, while no abnormalities were found in polycythemia vera. This may merely reflect the extensiveness of the extramedullary involvement in these syndromes. Although gamma globulins were abnormal in patients of the myeloproliferative category, isoagglutinin titers did not reflect these abnormalities, but showed a normal distribution.

Acute leukemia is also associated with significant serum protein alterations. The presence of hypoalbuminemia, hypoproteinemia, and increased gamma globulin in the present series confirms the results of other investigators.[5, 6, 10, 11, 35] These changes revert toward normal during remission.

Our results in multiple myeloma are also similar to those of many authors.[14-26] Hypoalbuminemia is associated with an elevation of alpha-1 and gamma globulins and an increase in total protein concentration. All the myeloma proteins found had mobilities in the gamma area. It should be noted, however, that myeloma proteins may occur in the range from alpha-2 through slow gamma.

Therapy led to a partial reversion of the protein pattern to normal with improvement of the clinical status. Such a change has also been reported by

Rundles and associates.[36, 37] The complete, although transitory, disappearance of the myeloma protein band noted in one of our patients is, however, rare.

Although gamma globulins were increased in myeloma, a decrease in isoagglutinin titers suggested that there was an actual decrease in the proportion of normal gamma globulin components.

It is noteworthy that homogeneous serum components may also be found in diseases other than myeloma.[8, 24, 38-47] In the present series, 3 cases (1 with lymphocytic lymphoma, 1 with chronic lymphocytic leukemia, and 1 with polycythemia vera) had peaks in the gamma range resembling myeloma protein. One might postulate that the malignant lymphocyte is the source of this protein in the first 2 cases, since the close relationship between plasma cell, lymphocytes, and gamma globulins has been emphasized by many.[38, 48-56] The source of the abnormal protein in the third case remains unexplained.

Significantly decreased isoagglutinin titers occurred in our patients with malignant lymphoma, chronic lymphocytic leukemia, and multiple myeloma. Schiff and Mendlowicz[57] were the first investigators to record the frequency of very low isoagglutinin titers in leukemia. Zuendel[58] corroborated these observations in another series of leukemic patients. He also noted that titers increased with remission of the disease. Davidsohn studied 38 patients with acute and chronic leukemia and found that the titers in the chronic cases were less than those of a control group. He noted no difference between myelocytic and lymphocytic leukemia, but he found both to be more significantly decreased than the acute cases. It is possible that the tendency toward low values could be partially explained in our series by the older age incidence in the lymphocytic leukemia and myeloma group, since the titers are said to decline with age.[59] However, the trend was not confined to older patients.

Several patients with hypogammaglobulinemia suffered bacterial infections during the course of the study. The association of the lack of immune response with recurrent infections in patients with malignant disorders of the hematopoietic and reticuloendothelial system has been emphasized,[60-66] and the low titers of isoagglutinins in some of these diseases may be considered as another manifestation of the inadequate immune mechanisms often present in these disorders. Recognition of this defect could certainly be of some clinical significance in the management of these cases.

SUMMARY

The serum proteins in 99 patients with Hodgkin's disease, other malignant lymphomas, acute leukemia, chronic lymphocytic leukemia, the myeloproliferative disorders, and multiple myeloma have been studied by electrophoretic and serologic techniques. The results of this analysis have been described, and the significant alterations commented upon together with observations on the effects of therapy. In addition, the occurrence of myeloma-like proteins in the sera of 3 patients with lymphoblastoma, chronic lymphocytic leukemia, and polycythemia rubra vera is reported.

We are grateful to Dr. W. C. Moloney for his encouragement and helpful criticism throughout this study. We are indebted to Miss Dalila Seminavicius for valuable technical assistance.

REFERENCES

1. Nitshe, G. A., Jr., and Cohen, P. P.: Serum Protein Changes in Myelogenous and Lymphocytic Leukemias and Hodgkin's Disease, Blood **2:** 363, 1947.
2. Petermann, M. L., Karnofsky, D. A., and Hogness, K. R.: Electrophoretic Studies on the Plasma Proteins of Patients With Neoplastic Disease. III. Lymphomas and Leukemia, Cancer **1:** 109, 1948.
3. Rottino, A., Suchoff, D., and Stern, K. G.: Electrophoretic Study of the Blood Serum From Lymphogranulomatous Patients, J. LAB. & CLIN. MED. **33:** 624, 1948.
4. Brown, R. K., Read, J. T., Wiseman, B., and Frand, W. G.: The Electrophoretic Analysis of the Serum Proteins of the Blood Dyscrasias, J. LAB. & CLIN. MED. **33:** 1522, 1948.
5. Gutter, F. J., Krevans, J. R., Moulton, G. A., and Kegeles, G.: Electrophoretically Determined Blood Protein Levels in a Case of Acute Myelogenous Leukemia Under Exchange Transfusion Treatment, J. Nat. Cancer Inst. **12:** 323, 1951.
6. Chury, Z., and Wiedermann, D.: Examination of Blood Proteins in Leukemia by Paper Electrophoresis, Scripta med. **27:** 112, 1954.
7. Arends, T., Coonrad, E. V., and Rundles, R. W.: Serum Proteins in Hodgkin's Disease and Malignant Lymphoma, Am. J. Med. **16:** 833, 1954.
8. Rundles, R. W., Coonrad, E. V., and Arends, T.: Serum Proteins in Leukemia, Am. J. Med. **16:** 842, 1954.
9. Gigante, D., Rossi-Espagnet, A., Capone, M., and Marinoni, G. F.: Studi sulle proteine del siero nelle emopatie maligne. Nota IV. II quadro sieroproteico nella mielosi leucemia cronica, Progr. méd. Paris **11:** 225, 1955.
10. Gigante, D., Rossi-Espagnet, A., Capone, M., and Marinoni, G. F.: Studi sulle proteine del siero emopatie maligne. Nota V. II quadro sieroproteico nella leucemia acuta, Progr. méd. Paris **11:** 289, 1955.
11. Rebello, E.: L'electrophorese dans les lymphomes, Acta Unio internat. contra cancrum **11:** 302, 1955.
12. Olmes, J., Mangin, M., and Martin, M.: Interet de l'electrophorese sur papier dans les leucoses, les reticulopathies malignes et dans l'individualisation des "dysglobulinemies primitives," Presse méd. **64:** 101, 1956.
13. Creysel, R., Morel, P., Vieux, R., Pechat, A., Croizat, P., and Revol: L'electrophorese du serum su cours des hemopathies malignes, Sang **28:** 34, 1957.
14. Kekwick, R. S.: Serum Proteins in Multiple Myelomatosis, Biochem. J. **34:** 1248, 1940.
15. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, J., and Kabat, E. A.: Fractionation of Serum Proteins in Hyperproteinemia With Special Reference to Multiple Myeloma, J. Clin. Invest. **20:** 765, 1941.
16. Adams, W. S., Alling, E. L., and Lawrence, J. S.: Multiple Myeloma. Its Clinical and Laboratory Diagnosis With Emphasis on Electrophoretic Abnormalities, Am. J. Med. **6:** 141, 1949.
17. Rundles, R. W., Cooper, G. R., and Willett, R. W.: Multiple Myeloma. IV. Abnormal Serum Component and Bence Jones Protein, J. Clin. Invest. **30:** 1125, 1950.
18. Lewis, L. A., Bortz, D. W., and Battle, J. D.: Electrophoretic and Ultracentrifugal Analysis of Serum Proteins in Multiple Myeloma, Cleveland Clin. Quart. **18:** 119, 1951.
19. Waldenstrom, J.: Abnormal Proteins in Multiple Myeloma. In: Advances in Internal Medicine, edited by I. Snapper, and W. Dock, Chicago, 1952, Year Book Publishers, Inc., vol. V.
20. Putnam, F. W., and Udin, B.: Proteins in Multiple Myeloma. I. Physicochemical Study of Serum Proteins, J. Biol. Chem. **202:** 727, 1953.
21. Reiner, M., and Stern, K. G.: Electrophoretic Studies on the Protein Distribution in the Serum of Multiple Myeloma Patients, Acta haemat. **9:** 19, 1953.
22. Griffiths, L. L., and Brews, V. A.: The Electrophoretic Pattern in Multiple Myelomatosis, J. Clin. Path. **6:** 187, 1953.
23. Conn, H. O., and Klatskin, G.: Filter Paper Electrophoretic Patterns of Serum in Multiple Myeloma, Am. J. Med. **16:** 822, 1954.
24. Osserman, E. F., and Lawlor, D. P.: Abnormal Serum and Urine Proteins in Thirty-five Cases of Multiple Myeloma, as Studied by Filter Paper Electrophoresis, Am. J. Med. **18:** 462, 1955.
25. Jim, R. T. S.: An Electrophoretic Study of the Abnormal Proteins in Urine, Peripheral Venous and Blood Marrow Sera in Multiple Myeloma, Blood **12:** 56, 1957.
26. Combined Staff Clinic. Multiple Myeloma. Current Clinical and Chemical Concepts, Am. J. Med. **23:** 283, 1957.

27. Davidsohn, I.: Isoagglutinin Titers in Serum Disease, in Leukemias, in Infectious Mononucleosis, and After Blood Transfusions, Am. J. Clin. Path. **8**: 179, 1938.
28. Williams, F. G., Pickels, E. G., and Durrum, E. L.: Improved Hanging Strip Paper Electrophoresis Technique, Science **121**: 829, 1955.
29. Jencks, W. P., Durrum, E. L., and Jetton, M. R.: Paper Electrophoresis as a Quantitative Method: Serum Proteins, Biochem. J. **60**: 205, 1955.
30. Franglen, G. T., and Martin, N. H.: The Interaction of Dyes With Proteins on Paper With Special Reference to Paper Electrophoresis, Biochem. J. **57**: 626, 1954.
31. Crook, E. M., Harris, H., Hassan, F., and Warren, F. L.: Continuous Direct Photometry of Dyed Materials in Filter Paper With Special Reference to the Estimation of Proteins Separated by Electrophoresis, Biochem. J. **56**: 434, 1954.
32. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F.: Studies in Serum Proteins. V. A Rapid Procedure for the Estimation of Total Protein, True Albumin, Total Globulin, Alpha Globulin, Beta Globulin and Gamma Globulin in 1.0 Ml. of Serum, Am. J. Clin. Path. **18**: 723, 1948.
33. Hill, A. B.: Principles of Medical Statistics, New York, 1955, Oxford University Press.
34. Conn, H. J.: Biological Stains, Geneva, New York, 1936, W. F. Humphrey Press, Inc.
35. Jencks, W. P., Smith, E. R. B., and Durrum, E. L.: The Clinical Significance of the Analysis of Serum Protein Distribution by Filter Paper Electrophoresis, Am. J. Med. **21**: 387, 1956.
36. Dillon, E. S., Dillon, M. L., and Rundles, R. W.: Changes in Electrophoretic Patterns of Sera in Patients With Multiple Myeloma Treated With Urethane, Am. J. Med. **7**: 242, 1949.
37. Rundles, R. W., Dillon, M. L., and Dillon, E. S.: Multiple Myeloma-Effect of Urethane Therapy on Plasma Cell Growth, Abnormal Serum Protein Components and Bence-Jones Proteinuria, J. Clin. Invest. **29**: 1243, 1950.
38. Bing, J., and Plum, P.: Serum Proteins in Leucopenia, Acta med. scandinav. **92**: 415, 1937.
39. Abrams, A., Cohen, P. P., and Meyer, O. O.: The Physical Properties of a Cryoglobulin Obtained From Lymph Nodes and Serum of a Case of Lymphosarcoma, J. Biol. Chem. **181**: 237, 1949.
40. Schwartz, T. B., and Jager, B. V.: Cryoglobulinemia and Raynaud's Syndrome in a Case of Chronic Lymphocytic Leukemia, Cancer **2**: 319, 1949.
41. Dalgaard, E. B.: A Case of Hyper- and Macro-globulinemia Accompanied by Atypical Lymphatic Hyperplasia, Acta path. et microbiol. scandinav. **27**: 506, 1950.
42. McFarlane, A. S., Davey, A., Slack, H. G. B., and Papastamatis, S. C.: An Unusual Case of Hyperglobulinemia, J. Path & Bact. **64**: 335, 1952.
43. Craig, A. B., Waterhouse, C., and Young, L. E.: Autoimmune Hemolytic Disease and Cryoglobulinemia Associated With Chronic Lymphocytic Leukemia, Am. J. Med. **13**: 793, 1952.
44. Henchel, G., and Eitner, H.: Über besondere bluteweife befunde Abei retothelsarkomatose, Acta haemat. **12**: 27, 1954.
45. Shaw, G.: Bence-Jones Proteinuria and Hypergammaglobulinemia in a Case of Renal Carcinoma, Glasgow M. J. **35**: 79, 1954.
46. Osserman, E. F., and Gellhorn, A.: A Postulated Protein Link Between Multiple Myeloma and the Lymphomas, Proc. Am. A. Cancer Res. **2**: 38, 1955.
47. Azar, H. A., Hill, W. T., and Osserman, E. F.: Malignant Lymphoma and Lymphatic Leukemia Associated With Myeloma-Type Serum Proteins, Am. J. Med. **23**: 239, 1957.
48. Good, R. A., and Varco, R. L.: A Clinical and Experimental Study of Agammaglobulinemia, Lancet **75**: 245, 1955.
49. Bing, J.: Further Investigations in Hyperglobulinemia, Acta med. scandinav. **103**: 547, 1940.
50. Keuning, F. J., and van der Slikke, L. B.: The Role of Immature Plasma Cells, Lymphoblasts, and Lymphocytes in the Formation of Antibodies, as Established in Tissue Culture Experiments, J. Lab. & Clin. Med. **36**: 167, 1950.
51. Gomsen, H.: Investigations on the Role of Plasma Cells as Antibody Producers, Sang **21**: 483, 1950.
52. Gupta, N. P., and Mangalik, V. S.: Plasma Cells and Antibody Production, Indian J. M. Res. **41**: 319, 1953.
53. Kass, E. H.: The Occurrence of Normal Serum Gamma Globulin in Human Lymphocytes, Science **101**: 337, 1945.
54. Dougherty, T. F., White, A., and Chase, J. H.: Relationship of Antibody Content of Normal and Malignant Lymphocytes, Proc. Soc. Exper. Biol. & Med. **59**: 172, 1945.
55. Ehrich, W. E., and Harris, T. N.: The Site of Antibody Formation, Science **101**: 28, 1945.
56. Harris, T. N., and Harris, S.: The Genesis of Antibodies, Am. J. Med. **20**: 114, 1956.

57. Schiff, F., and Mendlowicz, L.: Quantitativ untersuchungen über die emphendlichkeit menshlichter erythrozyten fur isogglutinine, Ztschr. Immunitätsforsch **45**: 207, 1925.
58. Zuendel, W.: Blutgruppenbeshemungen spezull bei blutkrankheiten, Klin. Wchnschr. **12**: 1872, 1933.
59. Thomsen, O., and Kettel, K.: Die staerke der menschlichen iso -agglutinine und entisprechenden blutkörperchenrezeptoren in berschienderen lebensaltern, Ztschr. Immunitätsforsch **63**: 67, 1949.
60. Wise, N. B., and Poston, M. A.: The Co-existence of Brucella Infections and Hodgkin's Disease, a Clinical, Bacteriologic, and Immunologic Study, J. A. M. A. **115**: 1976, 1940.
61. Dubin, I. N.: The Poverty of the Immunological Mechanism in Patients With Hodgkin's Disease, Ann. Int. Med. **27**: 998, 1947.
62. Evans, R. W.: The Antibody Response in Cases of Radiation Lymphopenia, and in the Reticuloses, J. Path. & Bact. **60**: 123, 1948.
63. Larson, D. L., and Tomlinson, L. J.: Quantitative Antibody Studies in Man. III. Antibody Response in Leukemia and Other Malignant Lymphomata, J. Clin. Invest. **32**: 317, 1953.
64. Marks, J.: Antibody Formation in Myelomatosis, J. Clin. Path. **6**: 62, 1953.
65. Zinneman, H. H., and Hall, W. H.: Recurrent Pneumonia in Multiple Myeloma and Some Observations on Immunologic Response, Ann. Int. Med. **41**: 1152, 1954.
66. Lawson, H. A., Stuart, C. A., and Paul, A. M.: Observations on the Antibody Content of the Blood in Patients With Multiple Myeloma, New England J. Med. **252**: 13, 1955.

THE ACID-BASE BALANCE AND SODIUM DISTRIBUTION OF THE BLOOD IN ESSENTIAL HYPERTENSION

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ACUTE alterations of the partial pressure of carbon dioxide within the pulmonary alveoli affect the vascular system. Respiratory acidosis produced by the inhalation of carbon dioxide causes an elevation of both systolic and diastolic blood pressures of normal man.[1] The normal dog and the renal hypertensive dog have a similar pressor response, although the neurogenic hypertensive dog and the curarized dog show a depressor effect.[2]

The converse of acute hypercapnea causing elevation of the blood pressure has also been reported, that is, it has been found that in essential hypertension there is a primary respiratory acidosis.[3] It is possible that this may be related to the alteration in the serum sodium concentration which has been reported in this disease.[4, 5] These considerations prompted further study of the acid-base balance and the plasma and whole blood sodium and water contents of patients having essential hypertension. Preliminary studies tended to confirm the reported association of respiratory acidosis with essential hypertension.* Further evaluation has failed to substantiate this correlation.

MATERIALS AND METHODS

Eleven normotensive subjects, 8 laboratory workers and 3 patients (9 men and 2 women), with resting blood pressures below 145/90 mm. Hg and 26 patients (16 men and 10 women) with essential hypertension having blood pressures above these levels were studied. Five of the hypertensive subjects were receiving antihypertensive drugs (3 reserpine and 2 mecamylamine). None had clinical or laboratory evidences of pulmonary disease, cardiac failure, or renal insufficiency. Arterialized, heparinized venous blood was withdrawn from the heated arm after 30 minutes of recumbency and the plasma separated anaerobically. Blood pressure readings were taken before and after venesection. The mean blood pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. Plasma pH,[6] CO_2 content,[7] and the per cent of packed red cells were determined, and the partial pressure of carbon dioxide (pCO_2), the plasma bicarbonate concentration, and the whole blood buffer base concentration (B_B') were calculated.[8] The water content of both plasma and whole blood was determined by drying to constant weight and the sodium concentration by flame photometry.

RESULTS

The mean blood pressure of the normal group was 90 mm. Hg and that of the hypertensive patients was 146 mm. Hg (Table I). In the hypertensive

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Supported in part by a grant from the American Heart Association.

Received for publication July 28, 1958.

*Abstracted in the Proceedings of the Central Society for Clinical Research, J. Lab. & Clin. Med. 50: 964, 1957.

group the male patients had a mean pressure of 148 mm. Hg and the female subjects had a mean of 142 mm. Hg. The hypertensive patients had a lower mean hematocrit value than did the normotensive subjects. This was not due to the proportionally greater number of female patients in this group because the mean hematocrit values for each sex were nearly the same. The plasma pH was similar in both groups, being 0.02 pH unit lower in the hypertensive patients. There were virtually no differences in the mean plasma CO_2 contents or calculated bicarbonate concentrations. The whole blood buffer base concentration (B_{B^+}) was slightly lower in the hypertensive group because of the lower per cent of red cells present. The partial pressure of carbon dioxide in the arterialized blood (pCO_2) was slightly higher in the hypertensive group. In the normotensive group the male subjects had a mean pCO_2 of 44.9 mm. Hg and the females 43.9 mm. Hg. In the hypertensive group the male patients had a mean pCO_2 of 44.9 mm. Hg and the females 46.4 mm. Hg.

TABLE I. MEAN DETERMINED AND DERIVED VALUES DESCRIBING THE ACID-BASE BALANCE

GROUP	MEAN BLOOD PRESSURE (mm. Hg)	HEMATOCRIT (PER CENT)	PLASMA pH (UNITS)	PLASMA CO_2 CONTENT (mM./L.)	WHOLE BLOOD (B_{B^+}) (mEq./L.)	pCO_2 (mm. Hg)
Normal						
Mean	90	47.8	7.38	26.84	48.4	44.7
S.E.		1.08	0.009	0.432	0.379	1.13
Hypertensive						
Mean	146	44.2	7.36	26.89	47.6	45.5
S.E.		0.96	0.009	0.511	0.384	1.31

S.E. = Standard error of the mean.

TABLE II. MEAN DETERMINED AND DERIVED VALUES DESCRIBING SODIUM CONTENT AND DISTRIBUTION

GROUP	PLASMA SODIUM (mEq./L.)	PLASMA WATER (GM./100 GM.)	WHOLE BLOOD SODIUM (mEq./L.)	WHOLE BLOOD WATER (GM./100 GM.)	RED CELL SODIUM (mEq./L. R.B.C.)
Normal					
Mean	143	91.8	92	79.1	35
S.E.	1.22	0.75	2.68	0.75	6.24
Hypertensive					
Mean	142	91.2	93	80.3	34
S.E.	1.30	0.15	2.19	0.49	6.08

The mean plasma sodium concentration of the hypertensive group was 1 mEq. lower than that of the normotensive subjects whereas the mean whole blood sodium concentration was 1 mEq. higher (Table II). The calculated sodium content per liter of red cells was slightly lower in the hypertensive patients. The plasma water content was nearly the same in both groups. The whole blood water content was slightly higher in the hypertensive group leading to a greater calculated cell water content per liter of red cells. When the sodium concentration of the plasma and red cells is calculated per liter of water in each phase, the hypertensive group had a lower intracellular sodium concentration.

None of the differences between group means in the parameters of the acid-base balance or in sodium distribution was statistically significant. Graphic presentation of these determinants disclosed no correlations with the blood pressure.

DISCUSSION

Waldron and Goldstein reported that the mean pH of the arterial blood of 19 hypertensive patients without renal functional impairment was 0.05 unit lower than that of 14 normotensive subjects and their mean partial pressure of carbon dioxide (pCO_2) was nearly 8 mm. Hg higher.[3] Although these differences in pH and pCO_2 between groups were statistically significant, all of these values fall within the normal range as defined by Singer and Hastings.[8] From the data of Waldron and Goldstein, assuming the pH's to be corrected to body temperature and the normal arterial oxygen capacity to be 19.0 vol. per cent with an hematocrit of 45 per cent, the mean whole blood buffer base is calculated to be 48.6 mEq./L. in the normotensive subjects and 47.5 mEq./L. in the hypertensive patients, both being normal values.

These findings in the hypertensive group of a lower pH, a higher pCO_2, and an unaltered buffer base are in the direction of those present in a primary respiratory acidosis but without any associated metabolic deviation in the acid-base balance. The data reported in this study are in agreement with the findings of Waldron and Goldstein as to direction of change but the differences between groups are of lesser magnitude and lack statistical significance.

Many factors influence the carbon dioxide tension and pH of the arterial blood. Variations due to diet, activity, and age were eliminated in the study of Waldron and Goldstein. Sex distribution was not specified. It has been found that the CO_2 tension of the blood is 4 mm. Hg less in women than in men.[9] In the series presented here this relationship was not found in the hypertensive patients. The pCO_2 of the female patients was slightly higher than that of the male subjects. This suggests that the female hypertensive patients do have a tendency toward a primary respiratory acidosis while the male subjects with hypertension do not. From this evidence it would appear that there is no direct relationship between the primary respiratory acidosis and the presence of essential hypertension per se.

The acid-base balance of the blood is very sensitive to changes in respiratory activity. During sleep the carbon dioxide tension of the blood rises about 7 mm. Hg.[10] Similarly a primary respiratory acidosis ensues quite promptly following initiation of breathing into a closed system.[11] In such acute situations there is no associated metabolic alteration in the blood buffer base. If a primary change in the acid-base balance persists long enough a secondary change in the other variable does occur. Such is the case in pulmonary emphysema where the primary respiratory acidosis is accompanied by a secondary metabolic alkalosis.[8] The absence of a secondary alteration in the blood buffer base in the hypertensive patients studied by Waldron and Goldstein suggests that the tendency toward a primary respiratory acidosis was of relatively short duration and therefore likely due to a transient

physiologic disturbance rather than to pulmonary disease. Indeed these authors state that their patients were free of any pathologic condition which might interfere with normal pulmonary ventilation.[3] In the hypertensive subjects reported in the present study there was likewise no evidence of pulmonary emphysema or any other pulmonary disease.

The hypernatremia reported to be present in hypertensive patients as a group was not demonstrated in this study. Holley and Holland found the mean plasma sodium concentration of 75 hypertensive patients to be 2.5 mEq./L. higher than that of 400 normotensive subjects.[4] Albert and coworkers reported that the plasma sodium concentration was nearly 6 mEq./L. higher in hypertensive subjects.[5] A previous study from this laboratory suggested that the milder cases of essential hypertension had slightly elevated plasma sodium concentrations.[12] This was not evident in the survey presented here.

SUMMARY

A study of 26 patients having essential hypertension and 11 normotensive subjects failed to demonstrate a direct relationship between elevation of the blood pressure and the presence of a primary respiratory acidosis or hypernatremia. The tendency of hypertensive patients as a group to have a respiratory acidosis, as evidenced by the slight elevation of the partial pressure of carbon dioxide and lowering of the pH of their blood, but without any change in buffer base, is most likely the result of transient physiologic hypoventilation.

REFERENCES

1. Dripps, R. D., and Comroe, J. H., Jr.: The Respiratory and Circulatory Response of Normal Man to Inhalation of 7.6 and 10.4% CO_2 With a Comparison of the Maximal Ventilation Produced by Severe Muscular Exercise, Inhalation of CO_2 and Maximal Voluntary Hyperventilation, Am. J. Physiol. **149:** 43-51, 1947.
2. Page, I. H., and Olmsted, F.: The Influence of Respiratory Gas Mixtures on Arterial Pressure and Vascular Reactivity in "Normal" and Hypertensive Dogs, Circulation **3:** 801-819, 1951.
3. Waldron, J. M., and Goldstein, F.: Changes in the Acid-Base Equilibrium in Essential Hypertension, J. Clin. Invest. **31:** 92-96, 1952.
4. Holley, H. L., Elliott, H. C., Jr., and Holland, C. M., Jr.: Serum Sodium Values in Essential Hypertension, Proc. Soc. Exper. Biol. & Med. **77:** 561-563, 1951.
5. Albert, D. G., Morita, Y., and Iseri, L. T.: Serum Magnesium and Plasma Sodium Levels in Essential Vascular Hypertension, Circulation **17:** 761-764, 1958.
6. Van Slyke, D. D., Weisiger, J. R., and Van Slyke, K. K.: Photometric Measurement of Plasma pH, J. Biol. Chem. **179:** 745-753, 1949.
7. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. **61:** 523-573, 1924.
8. Singer, R. B., and Hastings, A. B.: An Improved Clinical Method for the Estimation of Disturbances of the Acid-Base Balance of Human Blood, Medicine **27:** 223-242, 1948.
9. Shock, N. W., and Hastings, A. B.: Studies of the Acid-Base Balance of the Blood. III. Variations in the Acid-Base Balance of the Blood in Normal Individuals, J. Biol. Chem. **104:** 585-600, 1934.
10. Hastings, A. B., and Eisele, C. W.: Diurnal Variations in the Acid-Base Balance, Proc. Soc. Exper. Biol. & Med. **43:** 308-312, 1940.
11. Shock, N. W., and Hastings, A. B.: Studies of the Acid-Base Balance of the Blood. IV. Characterization and Interpretation of Displacement of the Acid-Base Balance, J. Biol. Chem. **112:** 239-262, 1935.
12. Cottier, P. T., Weller, J. M., and Hoobler, S. W.: Effect of an Intravenous Sodium Chloride Load on Renal Hemodynamics and Electrolyte Excretion in Essential Hypertension, Circulation **17:** 750-760, 1958.

ESTIMATION OF BILIRUBIN MONO- AND DIGLUCURONIDE IN THE PLASMA AND URINE OF PATIENTS WITH NONHEMOLYTIC JAUNDICE

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EVIDENCE from several laboratories indicates that the direct-reacting bilirubin in plasma is a mixture of two principal compounds, bilirubin mono- and diglucuronide.[1-4] The sole method available for estimating the bilirubin conjugates is the column chromatographic assay described by Billing.[1] With this technique Bollman[3] has observed that the estimation of bilirubin mono- and diglucuronide in plasma can be of value in the differential diagnosis of jaundice. Therefore, it seemed worth while to develop a more simple, rapid, and sensitive procedure for estimating the mono- and diglucuronide in direct-reacting bilirubin.

In the method described below the relatively labile bilirubin glucuronides are initially coupled with diazotized sulfanilic acid (the direct van den Bergh reaction) to yield the stable azopigment derivatives. In the reaction 1 mole of diglucuronide yields 2 moles of azopigment B, and 1 mole of monoglucuronide yields 1 mole of azopigment A plus 1 mole of azopigment B.[2] Subsequently, the azopigments are extracted, separated by a simple partition between butanol-chloroform and acetate buffer, and estimated colorimetrically. The proportions of mono- and diglucuronide in the direct-reacting bilirubin are calculated from the relative quantities of azopigments A and B found.

The method has been applied to estimations of the mono- and diglucuronide in the direct-reacting bilirubin in human T-tube bile, and in the plasma and urine of patients with nonhemolytic jaundice. In addition, the renal clearances of bilirubin mono- and diglucuronide have been determined simultaneously in each of 4 patients.

MATERIALS AND METHODS

Preparation of Azopigments.—Solutions of purified azopigments A and B were prepared as reference standards for the procedure. Azopigment A was prepared by coupling a chloroform solution of pure bilirubin (Mann Research Laboratories, m.p. 200° C., λ max. 450 mμ in chloroform, $E = 53.6 \times 10^3$) with diazotized sulfanilic acid in 50 per cent methanol.[5] The resulting solution of azopigment A was evaporated at 40° to 50° C. almost to dryness and diluted with water to a convenient volume. Azopigment B was prepared by treating fresh human T-tube bile with diazotized sulfanilic acid. Subsequently, the azopigment B was extracted with *n*-butanol and purified by ascending paper chromatography.[6] Alternatively, an equal volume of 2.0 M acetate of pH 3.0 was added

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This research was supported by a grant from the United States Public Health Service (A-1483).

Received for publication Aug. 6, 1958.

to the mixture of bile and diazo reagent, and the mixture was extracted with 4 volumes of *n*-butanol-chloroform (½, v/v) 3 times. The azopigment B remained in the acetate (upper) layer and could be used directly as a reference standard.

Preparation of Reagents.—Diazotized sulfanilic acid was freshly prepared.[5] The acetate buffer was prepared by adding 2.0 M sodium acetate to 2.0 M acetic acid until the mixture was exactly at pH 3.0 as determined with the Beckman glass electrode pH meter. Glycine buffer was similarly prepared by the dropwise addition of concentrated H_2SO_4 to 1.5 M glycine to exactly pH 2.0. The *n*-butanol and chloroform were Fisher certified reagents. The *n*-butanol was saturated with glycine buffer, and the chloroform was saturated with acetate buffer prior to use. Analytic reagent grade petroleum ether (b.p. 30° to 60° C.) was obtained from the Mallinckrodt Chemical Works.

Procedure.—Samples of blood and urine were obtained from 23 and 4 patients respectively, with nonhemolytic jaundice. Specimens of T-tube bile were collected from 4 patients following cholecystectomy. Heparinized venous blood samples were centrifuged immediately at 3,000 r.p.m. and 5° C. for 15 minutes to separate the plasma. All specimens were tested immediately.

Step 1. Formation of Azopigments: One milliliter of each sample of plasma, urine, or bile was treated with 0.5 ml. of diazotized sulfanilic acid in a glass-stoppered extraction tube. After 5 minutes the coupling reaction was stopped by the addition of 0.1 ml. of 0.2 M sodium azide, and 0.5 ml. of 1.5 M glycine of pH 2.0 was added. A control tube for each sample contained the same mixture except that the sodium azide was added before the diazo reagent. Aliquots of standard azopigments A and B added to water and to plasma were estimated simultaneously.

Step 2. Extraction of azopigments: Eight milliliters of *n*-butanol was added, the extraction tube stoppered, and the mixture shaken for 2 minutes by hand. After centrifugation at 2,000 r.p.m. in a clinical centrifuge, the clear supernatant layer of butanol contained 100 and 70 per cent, respectively, of the azopigments A and B.

Step 3. Separation and estimation of azopigments: In preliminary studies solutions of azopigments A (0.06 to 0.28 μM per milliliter) and B (0.26 to 0.50 μM per milliliter)* in 2.0 M sodium acetate of pH 3.0 were shaken for 2 minutes with 4 volumes of *n*-butanol-chloroform (½, v/v, saturated with the acetate buffer) in extraction tubes. The tubes were centrifuged for 1 minute at 2,000 r.p.m. in the clinical centrifuge. Subsequently the concentration of azopigment in each layer was estimated by the optical density at 540 mμ in the Beckman DU spectrophotometer in 3 ml. cuvettes with a 1.0 cm. light path. The acetate (upper) layer contained over 98 per cent of the azopigment B, while the *n*-butanol-chloroform (lower) layer contained 89 per cent of the azopigment A.

The azopigments in the butanol extract prepared in Step 2 were separated and estimated in a similar fashion. When the original sample contained 10 mg. per cent or more of direct-reacting bilirubin, 2.0 ml. of the butanol extract was pipetted into an extraction tube containing 4.0 ml. of chloroform and 1.5 ml. of 2.0 M acetate of pH 3.0. The tubes were shaken and centrifuged as just described, and the optical densities at 540 mμ of the acetate and butanol-chloroform layers were estimated.†

When the original sample contained 4 to 10 mg. per cent of direct-reacting bilirubin, 6.0 ml. of the butanol extract prepared in Step 2 was transferred to a 40 ml. extraction tube and shaken for 2 minutes with 12.0 ml. of chloroform and 4.5 ml. of 2.0 M acetate of pH 3.0.‡ After centrifugation at 1,500 r.p.m. for 5 minutes, 3.0 ml. of the acetate (upper) layer was removed for estimation of the optical density at 540 mμ. The azopigment A in the butanol-chloroform (lower) phase was concentrated by transferring 16.0 ml. of this layer to a 40 ml. extraction tube, and shaking with 4.0 ml. of 0.3 N NaOH and 16.0 ml. of

*Azopigment B is expressed in terms of Azopigment A as estimated by the optical density at 540 mμ.

†If the organic layer becomes turbid on standing, it can be cleared by holding the tube under the hot water tap for an instant and shaking the tube.

‡The ratio of acetate to butanol-chloroform is always 1:4.

petroleum ether for 2 minutes. The azopigment A was re-extracted into the 4.0 ml. of the aqueous (lower) layer, resulting in a fourfold increase in concentration. The optical density at 540 mμ of the azopigment A-containing phase was estimated after centrifugation.

Calculations.—The fraction (M) of direct-reacting bilirubin which is monoglucuronide, or diglucuronide (1-M), can be calculated from the concentrations of azopigment in the acetate buffer layer (C_A, expressed in optical density units per milliliter) and in the butanol-chloroform layer (C_O) estimated in Step 3, by the following equation*:

$$M = 6.27/(C_A/C_O + 2.66) \qquad (1)$$

When Equation 1 is rearranged to $C_A/C_O = 6.27(1/M) - 2.66$, the relationship between C_A/C_O and $1/M$ can be plotted linearly as shown in Fig. 1.

Comments.—The entire procedure was routinely completed within 30 minutes to minimize possible hydrolysis of azopigment B at pH 2 to 3. The values obtained were reproducible: in 10 determinations of a sample of plasma the per cent monoglucuronide in the direct-reacting bilirubin was 66 ± 2 (mean ± standard deviation).

The direct van den Bergh reaction as performed above appears to be specific for conjugated bilirubin. This was demonstrated by dissolving pure bilirubin in a small volume of 0.2 N NaOH and adding it to normal plasma. With concentrations of indirect-reacting bilirubin as high as 25 mg. per cent, the quantities of azopigment A formed in the direct reaction were insignificant.

RESULTS

Comparison With the Chromatographic Method.—The results of estimations of the per cent of mono- and diglucuronide in the direct-reacting bilirubin of bile, urine, and plasma are shown in Fig. 1, and agree with the values obtained by others with the column chromatographic method.[1, 3, 4] Bilirubin diglucuronide accounted for 84 and 81 per cent of the direct-reacting bilirubin in the bile and urine specimens, respectively. In contrast, bilirubin monoglucuronide was frequently the major fraction in the plasmas of the 23 patients with nonhemolytic jaundice. The mean value in plasma was 61 per cent monoglucuronide, comparable to 65 per cent monoglucuronide (19 observations, 17 patients) and 52 per cent (59 observations, 22 patients) reported in two studies with the chromatographic method.[1, 4]

A sample of plasma from each of 10 patients was tested by both the chromatographic and the present techniques to compare the methods directly. The values obtained paralleled each other with a correlation coefficient of 0.84 ($p < 0.01$). The proportion of monoglucuronide was generally 10 to 15 per cent lower with the chromatographic method.†

*$M = 2A/(A + B)$, where A = total azopigment A in the acetate/butanol-chloroform tube = (C_O) (6.0) (1/0.89), and B = total azopigment B in the tube = $\overline{(C_A)\ (1.5) - (A)\ (0.11)}$ (1/0.7). Substituting for A and B and simplifying results in Equation 1. The factor 1/0.7 corrects for the less complete extraction of azopigment B relative to azopigment A in the initial butanol extraction. The values for the constants in Equation 1 should be checked with standard azopigments A and B, and may vary slightly.

†Billing, Cole, and Lathe[2] have noted a requirement for ethanol for complete coupling of pigment I (obtained from columns) with the diazo reagent. If this were also true in plasma, it would result in low values for the monoglucuronide as estimated above. However, the monoglucuronide values are somewhat higher than those observed with the column method, suggesting that in plasma the bilirubin monoglucuronide may be coupled completely with diazotized sulfanilic acid in the absence of ethanol.

Plasma Bilirubin Glucuronides.—Table I summarizes the results of estimations of the per cent monoglucuronide in the plasmas of 23 jaundiced patients with various diagnoses. In each instance the diagnosis was established by history and physical examination, liver function tests, typical clinical course, and, except in the cases of probable viral hepatitis, by liver biopsy or surgical exploration. The per cent monoglucuronide ranged from 55 to 65 in 13 of the

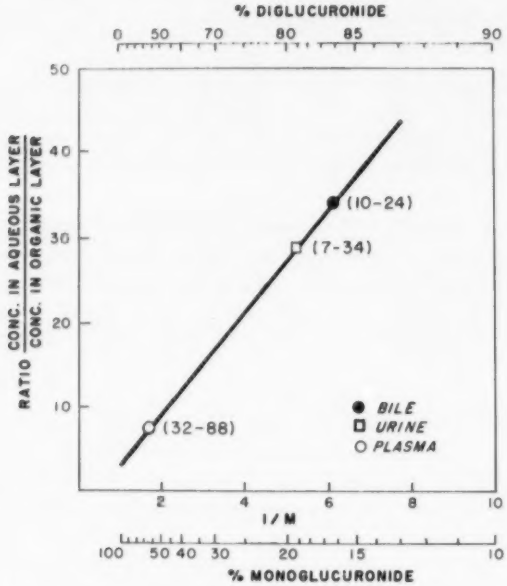

FIG. 1.—Relationship between the ratio of the concentrations of azopigments in the aqueous and organic layers and the proportions of mono- and diglucuronide in the direct-reacting bilirubin. A plot of C_A/C_O versus 1/M (see text) results in the straight line shown. The mean values (indicated on the line) and the ranges of values for the per cent monoglucuronide (figures in parentheses) in bile, urine, and plasma are shown.

TABLE I. THE PER CENT OF MONOGLUCURONIDE IN THE PLASMA DIRECT-REACTING BILIRUBIN OF PATIENTS WITH NONHEMOLYTIC JAUNDICE

NUMBER OF PATIENTS	PER CENT OF TOTAL PATIENTS	PER CENT MONO-GLUCURONIDE	DIAGNOSES*
4	17	32-48	Acute choledocholithiasis (1); early viral hepatitis (1); cholangiolitic hepatitis (1); hemochromatosis (1)
13	57	55-65	Viral hepatitis (9); chronic biliary obstruction [congenital atresia of bile ducts (1); carcinoma of common bile duct (1); carcinoma of head of pancreas (1)]; portal cirrhosis (1)
6	26	67-88	Portal cirrhosis (2); diffuse hepatic carcinoma (2); secondary biliary cirrhosis, 8 years (1); Dubin-Johnson syndrome (1)

*The number of cases with each diagnosis is indicated by parentheses.

23 patients. This group included most of the cases of viral hepatitis and 3 patients with extrahepatic biliary obstruction for over 2 months. Relatively low monoglucuronide values (i.e., high diglucuronide levels) were observed in individual patients with acute choledocholithiasis (32 per cent monoglucuronide), viral hepatitis at the onset of jaundice (43 per cent), cholangiolitic hepatitis (48 per cent), and hemochromatosis (45 per cent). Relatively high monoglucuronide levels, ranging from 67 to 88 per cent, were observed in 6 patients, including 2 cases of portal cirrhosis (67 and 69 per cent), 2 cases of diffuse intrahepatic carcinoma (78 and 88 per cent), and 1 case of secondary biliary cirrhosis for over 8 years (74 per cent).

Renal Clearance of Bilirubin Glucuronides.—The renal clearances of bilirubin mono- and diglucuronide have been estimated simultaneously in each of 4 jaundiced patients. Timed, voided urine samples were collected for 2 to 3 hours, and venous blood samples were drawn at the mid-points of the collection periods. The value for the total direct-reacting bilirubin in each sample[5] was multiplied by the fraction of mono- and diglucuronide to yield the concentration of each conjugate in plasma and urine, and the renal clearances were calculated by conventional methods.[7] As shown in Table II the clearances of bilirubin diglucuronide varied from 0.41 to 0.96 ml. per minute, and considerably exceeded the clearances of the monoglucuronide, 0.05 to 0.16 ml. per minute.

TABLE II. THE RENAL CLEARANCES OF BILIRUBIN MONO- AND DIGLUCURONIDE

PATIENT	DIAGNOSIS	RENAL CLEARANCE (ML. PER MINUTE)	
		MONOGLUCURONIDE	DIGLUCURONIDE
1	Viral hepatitis	0.11	0.47
2	Biliary cirrhosis	0.16	0.88
3	Viral hepatitis	0.09	0.41
4	Carcinoma of head of pancreas	0.05	0.96

DISCUSSION

Experimental and clinical studies described by Bollman indicate that bilirubin is conjugated to the diglucuronide only in the liver. The latter compound is the major bilirubin conjugate secreted into the bile and may accumulate in the blood following acute biliary obstruction. Bilirubin monoglucuronide can be synthesized in extrahepatic tissues and may accumulate in the blood in hepatectomized animals and in patients with hepatic parenchymal disease.[3] Thus, it would be anticipated that estimation of the bilirubin glucuronides in plasma could be of value in the diagnosis of jaundice. In confirmation of Bollman's observations, high plasma diglucuronide levels were observed in a patient with acute common duct obstruction and in another with viral hepatitis during the onset of jaundice. The proportion of monoglucuronide was considerably higher in patients with chronic biliary obstruction (2 months or longer), and in these patients the proportion did not differ significantly from that observed in primary hepatic parenchymal disease. The highest monoglucuronide levels were noted in patients with advanced portal cirrhosis, diffuse hepatic neoplasm, and in one case of secondary biliary cirrhosis of 8 years' duration.

It seems reasonable to conclude that a marked predominance of bilirubin diglucuronide in the plasma suggests the diagnosis of acute biliary obstruction, whereas a very high proportion of the monoglucuronide suggests hepatic parenchymal disease. Inasmuch as almost 60 per cent of the patients studied comprised an intermediate group, with monoglucuronide levels varying from 55 to 65 per cent of the direct-reacting bilirubin, estimation of the glucuronides in plasma appears to be of limited diagnostic value.

The method of estimation described can be useful in studies of bile pigment metabolism. The procedure is simple, rapid, and applicable to samples containing as little as 4 mg. per cent direct-reacting bilirubin. The values obtained are similar to those observed with the more complicated chromatographic technique. In the present studies the method has been applied to the simultaneous estimation of the renal clearances of the bilirubin glucuronides. The predominance of the diglucuronide in the urine of patients with nonhemolytic jaundice[2, 8] is associated with a considerably higher renal clearance of this conjugate as compared to the monoglucuronide.

SUMMARY

1. A simple method is described for the estimation of the mono- and diglucuronide in the direct-reacting bilirubin of plasma, urine, and bile.

2. Bilirubin diglucuronide is the major bilirubin compound in human T-tube bile. In patients with nonhemolytic jaundice the diglucuronide predominates in the urine, whereas the monoglucuronide is frequently the major conjugate in the plasma.

3. Estimation of the bilirubin conjugates in plasma is of limited diagnostic value, although high diglucuronide levels suggest acute biliary obstruction and high monoglucuronide values suggest hepatic parenchymal disease.

4. The renal clearance of bilirubin diglucuronide is considerably greater than that of the monoglucuronide.

REFERENCES

1. Billing, B. H.: A Chromatographic Method for the Determination of the Three Bile Pigments in Serum, J. Clin. Path. **8:** 126, 1955.
2. Billing, B. H., Cole, P. G., and Lathe, G. H.: The Excretion of Bilirubin as a Diglucuronide Giving the Direct van den Bergh Reaction, Biochem. J. (London) **65:** 774, 1957.
3. Bollman, J. L.: Bile Pigments of Serum in Disease of Liver. In Hepatitis Frontiers, edited by F. W. Hartman, and associates, Henry Ford Hospital International Symposium, Boston, 1957, Little, Brown & Company, p. 467.
4. Baikie, A. G.: The Chromatographic Separation of the Serum Bile Pigments in Jaundice, Scott. Med. J. **2:** 359, 1957.
5. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119:** 481, 1937.
6. Schachter, D.: Nature of the Glucuronide in Direct-Reacting Bilirubin, Science **126:** 507, 1957.
7. Smith, H. W.: The Kidney. Structure and Function in Health and Disease, New York, 1951, Oxford University Press.
8. Schmid, R.: Direct-Reacting Bilirubin, Bilirubin Glucuronide, in Serum, Bile, and Urine, Science **124:** 76, 1956.

PLASMA RIBONUCLEASE ACTIVITY IN HUMAN UREMIA

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IT WAS previously shown that the activity of serum ribonuclease (RNase) increases in experimental animals after nephrectomy,[1] ureter ligation,[1, 2] or temporary constriction of the renal pedicles.[3] In the latter case, the enzyme activity increased in parallel fashion with the urea concentration of the serum. These findings suggested an investigation of the RNase activity in the blood of normal and uremic patients.

MATERIAL AND METHODS

The oxalated blood samples collected under fasting conditions were used for the determination of RNase[4] and urea. RNase activity was estimated in 5 μl samples of plasma as previously described,[1, 3] and urea was determined in whole blood by urease digestion followed by direct nesslerization.[5] RNase results are expressed in arbitrary optical density units. Under our assay conditions, 0.1 optical density unit is equivalent to the activity of 0.0012 μg crystalline bovine pancreatic RNase.

Table I. Ribonuclease Activity in Plasmas on Nonuremic Patients

Group	Number of cases	Average RNase activity*	Range	99 per cent confidence limits of mean
Miscellaneous (nontumoral) surgical conditions	15	0.094	0.020-0.203	0.055-0.133
Malignant tumors	11	0.151	0.053-0.262	0.107-0.215
Cardiovascular diseases	8	0.104	0.032-0.241	0.025-0.183
Infectious and parasitic diseases	6	0.132	0.054-0.194	0.040-0.224
Acute leukemia	4	0.076	0.047-0.115	0.000-0.164
Miscellaneous (nonleukemic) hematologic conditions	6	0.116	0.043-0.173	0.037-0.195
Diabetes	9	0.083	0.017-0.205	0.020-0.146
Nephrotic syndrome	11	0.127	0.045-0.265	0.050-0.204
Renal lithiasis	8	0.142	0.038-0.187	0.070-0.214
Pre-eclampsia	6	0.120	0.029-0.170	0.036-0.204
Acute glomerulonephritis	8	0.172	0.099-0.282	0.096-0.248
Other conditions	26	0.132	0.037-0.284	0.098-0.166
Total number of cases	118	0.122	95% confidence limits 0-0.248	
			99% confidence limits 0-0.292	

*Optical density per 10 μl of plasma.

RESULTS

The RNase values at normal blood urea levels were obtained from a sample of 118 subjects, 56 women and 62 men. Urea values up to 18.6 mg. BUN per

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This work was performed with the aid of grants from the Rockefeller Foundation and the Conselho Nacional de Pesquisas.

Received for publication Aug. 11, 1958.

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100 ml. were considered normal. In Table I this material is tentatively classified. In view of the high variability from subject to subject and the small number of cases studied, no emphasis is placed on the apparent nonhomogeneity of the normal urea subjects. For the present purposes the data were combined and the confidence limits calculated for descriptive purposes only, as the distribution of the enzyme activities does not seem to approach the normal one. A sample of 28 healthy medical students presented an average RNase activity 13 per cent lower than the "normal urea" sample (mean 0.105, range 0.016 to 0.227). This difference is barely significant at the 1 per cent probability level. When the normal urea cases were distributed according to sex, no significant difference attributable to this factor was found. Inspection of the data suggested that at normal urea levels no correlation apparently existed between blood urea and plasma RNase.

When "moderately" increased (18.6 to 46.6 mg. BUN per 100 ml.) blood urea levels were considered, a trend toward high plasma RNase activities was seen. These patients presented a variety of clinical conditions, and the cause of the uremia could not be ascertained in many of them. Judging from the history and clinical data, renal disease was present in 14 of 38 patients in this group. However, these 14 patients as a group did not behave differently from the other 24 as regards plasma RNase (Table II).

TABLE II. RIBONUCLEASE ACTIVITY IN THE PLASMA OF MODERATELY UREMIC PATIENTS (18.6 TO 46.6 MG. BUN PER 100 ML.)

GROUP	NUMBER OF CASES	AVERAGE BUN (MG./100 ML.)	RANGE	S.E.M.*	AVERAGE RNase	RANGE	S.E.M.*
Miscellaneous nonrenal patients	24	33	19-47	1.7	0.179	0.071-0.380	0.018
Renal patients	14	33	20-43	2.5	0.188	0.072-0.289	0.017

*S.E.M. = standard error of the mean.

Many of the patients who presented a high concentration of urea in the blood (above 46.6 mg. BUN per 100 ml.) also showed a distinct increase in plasma RNase activity. As can be seen in Table III and Fig. 1 Cases 20 to 23) the uremia was primarily of "extrarenal" origin and plasma RNase activity was in the upper normal range or slightly increased.

The results in uremic patients with renal disease showed that no strict correlation existed between blood urea and plasma RNase activities. Fig. 1 also shows that the increase in RNase activity was much smaller than that of urea. In a few instances (e.g., Case 5), RNase activity was remarkably stable over rather long periods of time, in spite of variations in the concentrations of urea in blood. In other patients (e.g., Cases 2 and 3), however, large fluctuations were found in RNase activity.

The cases studied do not permit a definite evaluation of the correlation between plasma RNase activity and the clinical outcome of the patients. It may be seen, however (Table III and Fig. 1), that the highest activities (Cases 1

and 2) belonged to irreversibly anuric subjects, maintained by means of repeated treatments with the artificial kidney.* Two other patients (Cases 3 and 4) were severely ill, and died soon after admission. On the other hand, the lower RNase activities belonged to patients with either acute renal disease with clinical recovery (Cases 16 and 18), or to stationary chronic renal disease with relatively low concentrations of urea in the blood (Cases 15 and 17). Cases

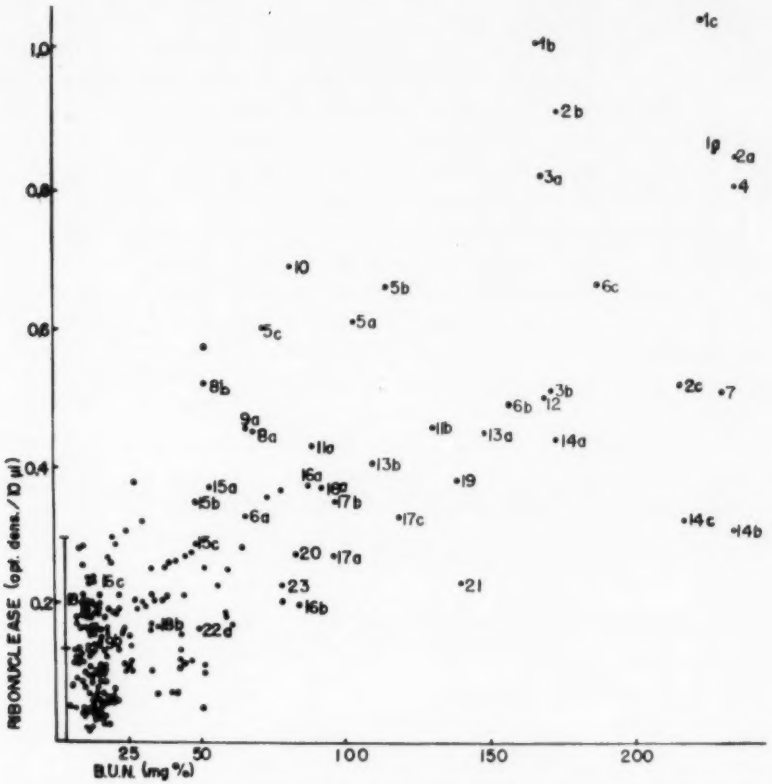

Fig. 1.—Plot of plasma RNase activity (in optical density units per 10 µl) against BUN (in milligrams per cent). The numbers in the figure pertain to the cases listed in Table III. A maximum of 3 pairs of values for each patient was plotted for the sake of clarity. The lettering refers to the first 3 successive samplings for each patient. On the vertical line are shown the average and the upper 99 per cent confidence limits of normal urea plasma RNase activity.

14 and 19, however, presented moderate increases in RNase activity in spite of a greater concentration of urea in blood and a fatal outcome, whereas Case 10 had a relatively high plasma RNase activity with a low degree of uremia. In the group with intermediate levels of RNase activities (Cases 5 to 13), some had

*It may be seen that dialysis had no effect on plasma RNase activity (Table III, Cases 1 and 2).

TABLE III. RIBONUCLEASE ACTIVITY IN BLOOD OF UREMIC PATIENTS WITH BLOOD UREA NITROGEN VALUES GREATER THAN 46.6 MG. PER CENT

CASE NO.	DIAGNOSIS*	PLASMA RNase†	DATE
1	Chronic renal disease; posttraumatic acute renal failure (C)	0.853	4/24
		1.010	4/28
		1.046	5/18(a)‡
		0.996	5/14(b)‡
2	Renal amyloidosis; acute renal failure post-surgery (C)	0.850	4/7
		0.912	4/10
		0.520	4/18(a)‡
		0.640	4/18(b)‡
		0.734	4/22
		0.751	4/23
3	Primary contracted kidney; malignant phase of hypertension (N)	0.820	4/24
		0.510	4/26
4	Chronic glomerulonephritis (N)	0.804	5/2
5	Nephrotic syndrome with advanced chronic renal failure (C)	0.610	4/10
		0.659	4/16
		0.606	4/23
		0.619	4/28
		0.613	5/3
		0.612	5/21
6	Renal tumor with urinary obstruction (C)	0.329	4/14
		0.526	4/16
		0.663	4/18
		0.625	4/22
7	Chronic glomerulonephritis (C)	0.512	
8	Chronic glomerulonephritis (C)	0.452	5/8
		0.525	5/22
9	Obstructive uremia, prostatic origin (C)	0.459	4/24
		0.124	5/6
10	Chronic kidney failure	0.513	5/17
11	Chronic kidney failure	0.435	4/24
		0.455	5/13
12	Chronic glomerulonephritis (N)	0.506	4/23
13	Chronic glomerulonephritis (C)	0.448	
		0.406	
14	Chronic renal failure; pionephrosis (N)	0.461	4/10
		0.313	4/16
		0.326	4/18
15	Chronic kidney disease (lupus erythematosus) (C)	0.372	4/12
		0.354	4/18
		0.293	4/23
16	Postabortum acute renal failure (C)	0.377	4/10
		0.202	4/14
		0.231	4/22
		0.158	5/2
17	Renal rickets (C)	0.277	4/14
		0.351	4/16
		0.328	4/18
		0.274	4/23
18	Acute glomerulonephritis (C)	0.373	5/2
		0.168	5/6
		0.199	5/8
19	Malignant phase, essential hypertension (N)	0.383	4/18
20	Postoperative vomiting	0.267	4/24
21	Liver cirrhosis (C)	0.235	4/23
		0.284	4/24
22	Postoperative vomiting	0.164	5/3
		0.123	5/6
		0.113	5/12
23	Generalized peritonitis (N)	0.227	5/21

*C = clinical; N = necropsy.
†Optical density per 10 μl of plasma.
‡(a) = After artificial kidney; (b) = before artificial kidney.

stationary but severe renal disease (Cases 5, 8, 10, 11, and 13) and others progressed rapidly to death (Cases 6, 7, and 12). Case 6 showed the progressive increase in both urea and RNase with impairment of renal function. Case 9 had a favorable course after prostatic obstruction was relieved, with a drop in both urea and RNase activity.

DISCUSSION

The above results show that plasma RNase activity increases in the blood of uremic patients. Extrarenal uremia does not seem to cause such increases, although it is recognized that our data are rather limited. Métais and Mandel[4] found no significant variation in serum RNase activity in the blood of 20 patients with several diseases, including patients with renal disease. No clinical or laboratory data were given that would permit further analysis of their results. Infections have been reported not to influence serum RNase activity.[6]

In a previous paper[3] we discussed the hypothesis that a decrease in the glomerular filtration rate could explain the increase in serum RNase activity found after temporary ligation of the renal pedicles in rats. A similar hypothesis might account for the increase in serum RNase activity found in uremic patients. However, more data are needed on the rate of production and release of the enzyme into the blood, and its mechanism of excretion in the urine[2, 3, 7] before such an hypothesis may be considered to be securely grounded.

The increase in RNase activity does not seem to be a consequence of uremia. Aside from the behavior of plasma RNase in the cases of extrarenal uremia reported here, this is borne out by experiments in dogs, in which ureterovenous anastomosis was performed; under these conditions, small variations in serum RNase were found, in spite of a NPN retention similar to that of binephrectomized animals.[8]

Much more experience with the method on more extensive clinical material is necessary before its possibilities as a diagnostic or prognostic tool may be evaluated.*

SUMMARY

Plasma RNase was estimated in blood samples of human subjects containing normal and high concentrations of urea. Marked increases in the plasma RNase were found coincident with a high urea concentration in patients with kidney impairment. No strict correlation was found, however, in different patients, between blood urea levels and plasma RNase activities.

The advice and facilities provided by Drs. J. B. Magaldi, I. Nussenzveig, A. Cardoso, and W. T. Beraldo and the technical assistance of Mrs. M. C. Cosentino are gratefully acknowledged. The blood urea determinations were performed by the staff of the Central Laboratory, Hospital das Clinicas.

*After this paper was prepared for publication our attention was called to the work of J. C. Houck and L. B. Berman (J. Appl. Physiol., 12: 473-476, 1958) who reported an increase in serum RNase activity in acute renal failure; these authors also presented data supporting a correlation between the white blood cell count and serum RNase activity.

REFERENCES

1. Rabinovitch, M., and Dohi, S. R.: Increase in Serum Ribonuclease After Bilateral Nephrectomy, Am. J. Physiol. **187**: 525-528, 1956.
2. Berman, L. B., and Houck, J. C.: Renal Clearance of Ribonuclease, Proc. Soc. Exper. Biol. & Med. **97**: 175-177, 1958.
3. Dohi, S. R., Fausto, N., Kipnis, J., Liberman, B., and Rabinovitch, M.: Serum Ribonuclease Activity After Temporary Ligation of the Renal Pedicles in Rats, Proc. Soc. Exper. Biol. & Med. (In press.)
4. Métais, P., and Mandel, P.: Activité ribonucléasique du serum humain, Bull. Soc. chim. biol. **37**: 999-1007, 1955.
5. Gentzkow, C. J., and Masen, J. M.: An Accurate Method for the Determination of Blood Urea by Direct Nesslerization, J. Biol. Chem. **143**: 531-554, 1942.
6. Aleksandrowicz, J.: Ribonuclease Activity in Chronic Granulocytic Leukaemia, Lancet **1**: 640-641, 1958.
7. Rabinovitch, M., and Dohi, S. R.: Aspectos bioquímicos e experimentais das ribonucleases sérica e urinária Ciência **9**: 195-200, 1957.
8. Dohi, S. R., Terzian, J. F., Widman, A., Brentani, R., Liberman, B., Fausto, N., and Rabinovitch, M.: Data to be published.

THE MECHANISM OF INSULIN ANTIDIURESIS

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INSULIN has been shown to produce antidiuresis independent of its effect upon the blood sugar concentration.[1, 2] It has been suggested that this antidiuresis is due to a specific effect of insulin upon water reabsorption by the renal tubule.[1] The present study was conducted to re-examine the mechanism of insulin antidiuresis and provides evidence that such antidiuresis is mediated through the action of antidiuretic hormone (ADH).

METHODS

Thirteen normal subjects, two patients with diabetes mellitus, and one patient with diabetes insipidus were studied during the forenoon while in a fasting, supine state. Measurements of glomerular filtration, rate of urine flow, and blood sugar concentration were made in all subjects. The glomerular filtration rate was measured by inulin clearance. The inulin infusion was administered at a constant rate by a constant infusion pump following an initial priming dose. Urine was collected through an indwelling catheter at 15-minute intervals and each collection period was terminated by saline and air washouts. Arterial blood samples for the determination of plasma inulin and blood glucose concentrations were obtained in heparinized syringes at the mid-point of each urine collection period through an indwelling needle. After 3 or 4 control urine collections were obtained, hyperglycemic factor-free (HGF) insulin was given rapidly into an antecubital vein and the 15-minute urine collections continued.

In the normal subjects water diuresis with rates of urine flow of 8 ml. per minute or greater was obtained by the oral administration of 800 to 1,000 ml. of water one hour prior to the study and was maintained by the administration of 700 ml. of water per hour during the study. In 6 of the normal subjects the intravenous administration of absolute alcohol was begun at a constant infusion rate of 340 mg. per minute 40 minutes prior to the beginning of the control urine collection periods and maintained for the duration of the experiment. None of the other individuals investigated received alcohol at any time during the study. All normal subjects who did not receive alcohol were given 0.15 to 0.20 unit HGF insulin per kilogram of body weight; all subjects who did receive alcohol were given 0.20 units HGF insulin per kilogram of body weight intravenously at the end of the control period.

The patients with diabetes mellitus had heavy glucosuria and had not received any type of insulin during the 24-hour period preceding the study. Water diuresis was produced in

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This investigation was supported in part by the Veterans Administration Clinical Investigator Program, in part by Research Grant A-1596 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and in part by the Life Insurance Medical Research Fund.

Received for publication Aug. 15, 1958.

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them by the same water loading protocol as used in the normal subjects. At the end of the control period, the patients with diabetes mellitus were given 30 and 40 units of HGF insulin intravenously.

The one patient with diabetes insipidus was responsive to aqueous Pitressin, and this drug had been withheld for a 3-day period immediately preceding the study. He was not given a water load, and received 0.30 unit HGF insulin per kilogram of body weight after control measurements were obtained.

All normal subjects and the patient with diabetes insipidus received glucose in the maintenance inulin infusion in order to prevent the development of hypoglycemia.

Inulin determinations were performed using Schreiner's modification of the resorcinol method.[3] Nelson's adaptation of the Somogyi method was used to determine blood glucose concentrations.[4]

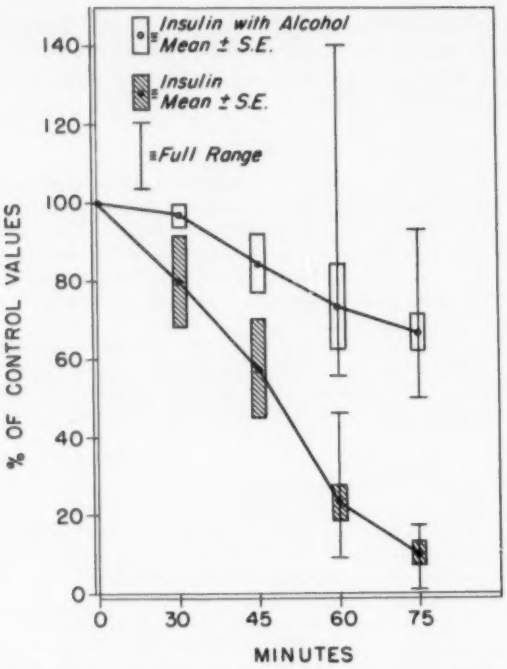

Fig. 1.—Diagram comparing the rate of urine flow in normal subjects who received insulin and in normal subjects who received insulin and alcohol intravenously. The abcissa denotes the time interval following insulin administration. Urine flows charted at zero time represent the means of the control values.

RESULTS

Control urine flows ranged from 8 to 13 ml. per minute in the normal subjects who did not receive alcohol and varied from 9 to 16 ml. per minute in the normal subjects who did receive alcohol. Insulin consistently produced an antidiuresis in the normal subjects who did not receive alcohol. This antidiuresis was associated with an increase of the urine specific gravity and occurred without a change in glomerular filtration rate. Alcohol administered prior to insulin

administration partially inhibited the development of antidiuresis (Fig. 1). In normal subjects who received only insulin, the rate of urine flow 75 minutes after insulin administration was 0.1 to 1.6 ml. per minute. In those normal subjects who received both insulin and alcohol, the range of urine flows obtained 75 minutes after insulin administration were 7.3 to 11 ml. per minute.

After insulin administration in the two patients with diabetes mellitus, antidiuresis was delayed until the disappearance of glucosuria 90 and 120 minutes later. None of the other subjects examined had glucosuria at any time during the experiment.

Administration of insulin to the patient with diabetes insipidus produced no change in urine flow or urine specific gravity (sp. gr. = 1.001). His control rates of urine flow were 6.7, 6.2, and 6.8 ml. per minute. Consecutive 15-minute collections after administration of insulin revealed urine flows of 6.7, 7.2, 7.8, 8.3, and 8.5 ml. per minute, respectively.

DISCUSSION

The failure of insulin to cause antidiuresis in a subject with diabetes insipidus responsive to Pitressin does not support the concept that insulin produces antidiuresis by a direct effect upon a renal tubular mechanism concerned with water reabsorption. Alcohol reportedly inhibits the release of ADH from the posterior pituitary.[5] The ability of alcohol to inhibit the antidiuretic response to insulin in normal subjects suggests that the antidiuresis caused by insulin administration is mediated through the action of ADH.

The inhibition of insulin antidiuresis by glucosuria (osmotic diuresis) in two subjects with diabetes mellitus is in keeping with this hypothesis.

It thus appears that the antidiuresis resulting from the administration of commercially available insulin may be mediated through the release of antidiuretic hormone.

SUMMARY

The antidiuresis in response to commercially available insulin is again confirmed. The failure of insulin to produce antidiuresis in a patient with diabetes insipidus and the inhibition of insulin antidiuresis in normal subjects by the administration of alcohol suggest that insulin antidiuresis is mediated through the action of ADH, and is not due to a specific effect of insulin upon the renal tubule.

REFERENCES

1. Miller, J. H., and Bogdonoff, M. D.: Antidiuresis Associated With Administration of Insulin, J. Appl. Physiol. **6**: 509-512, 1954.
2. Murdaugh, H. V., Jr.: Effects of Insulin Hypoglycemia on Renal Hemodynamics, J. LAB. & CLIN. MED. **48**: 69-75, 1956.
3. Schreiner, G. E.: Determination of Inulin by Means of Resorcinol, Proc. Soc. Exper. Biol. & Med. **74**: 117-120, 1950.
4. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375-380, 1944.
5. Van Dyke, H. B., and Ames, R. G.: Alcohol Diuresis, Acta Endocrinol. **7**: 110-121, 1951.

RENAL FACTORS RESPONSIBLE FOR THE HYPERMAGNESEMIA OF RENAL DISEASE

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FEW data are available describing the renal mechanisms of magnesium excretion either in normal man or in patients with renal disease. It is thought that magnesium is excreted in the urine of mammals as the net result of glomerular filtration and tubular reabsorption.[1] In addition, various authors have suspected that, as with potassium, tubular secretion of magnesium may occur.[2-5] Efforts to document the occurrence of tubular secretion have thus far been unsuccessful.[3-6]

It has been recognized for some time that many individuals with kidney disease may demonstrate hypermagnesemia at some point in the course of their disease.[7, 8] It has also been shown that the serum magnesium may be normal or low in such patients.[7] Thus far, no attempts have been made to compare the elevation of total serum magnesium with quantitative measurement of the changes in renal function which occur in patients with various types of renal disease. It was decided to determine whether any relationship existed between the total serum magnesium concentration and the degree of impairment of the glomerular filtration rate in individuals with renal disease.

MATERIALS AND METHODS

Eighteen men between the ages of 34 and 68 were studied. Their filtration rates varied between 9.0 to 93.0 ml. per minute and thus covered a wide range of impaired renal function in patients with kidney disease. Most of the subjects had either chronic pyelonephritis, chronic glomerulonephritis, or benign nephrosclerosis associated with hypertensive vascular disease. All patients received either regular hospital diets or diets that were restricted in sodium and/or protein according to the therapeutic demands of their illnesses. None received either abnormally high intakes of magnesium or any medication known to alter the serum magnesium concentration.

All studies were performed in the forenoon while the subjects were in the fasting, supine state. An adequate urine flow was obtained by the oral ingestion of 800 to 1,000 ml. of water approximately 30 to 60 minutes prior to beginning the study. Urine was collected by an indwelling catheter at intervals of 10 to 20 minutes. The bladder was

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This investigation was supported in part by funds from the United States Public Health Service (Grants H-2788, A-1596), and the Veterans Administration Clinical Investigator Program.

Received for publication Aug. 22, 1958.

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washed with 60 ml. of normal saline and 40 ml. of air at the end of each urine collection period. Venous blood was drawn without stasis through an indwelling No. 18 Cournand arterial-type needle into heparinized syringes at approximately the mid-point of each urine collection period. The blood samples were then centrifuged, and the plasma was withdrawn for the determination of inulin and magnesium concentrations.

The filtration rate was measured by the inulin clearance. The inulin was administered at a constant rate by a Bowman constant-infusion pump following an initial priming dose. The resorcinol method of Roe and associates as modified by Schreiner[9] was used for the determination of inulin in unyeasted urine and cadmium sulfate filtrates of plasma. A delay time of 2.5 minutes was allowed in the calculation of the inulin clearance.

Total serum magnesium concentration was determined in the trichloroacetic acid filtrate of plasma using a minor modification of the titan yellow method described by Garner.[10] The normal range in our laboratory as established by measurements made on 39 normal subjects is 1.7 to 2.2 mEq. per liter (mean: 1.93 ± S.D. 0.25 mEq. per liter). No estimation of the amount of protein-bound magnesium was made. Duplicate or triplicate determinations performed on the same sample were required to agree within ± 0.15 mEq. per liter.

Each series of observations on any one patient consisted of at least 4 consecutive urine collection periods.

TABLE I. GLOMERULAR FILTRATION RATE AND TOTAL SERUM MAGNESIUM CONCENTRATION IN PATIENTS WITH RENAL DISEASE

PATIENT	AGE	DIAGNOSIS	GFR (ML./MIN.)	SERUM MAGNESIUM (mEq./L.)
J. T.	63	Chronic pyelonephritis	9	2.98
S. M.*	40	Malignant nephrosclerosis	14	2.12*
J. S.	47	Chronic glomerulonephritis	16	3.10
C. B.	58	Chronic pyelonephritis	22	2.82
M. P.	64	Chronic pyelonephritis	25	2.62
C. K.	44	Malignant nephrosclerosis	27	2.67
H. M.	47	Chronic pyelonephritis	30	2.11
S. Y.	35	Benign nephrosclerosis	41	2.00
F. H.	36	Acute glomerulonephritis	59	1.50
J. W.	34	Acute glomerulonephritis	60	1.64
Z. J.	68	Chronic pyelonephritis, congestive heart failure	63	2.12
C. S.	61	Benign nephrosclerosis	71	2.07
F. A.	63	Chronic pyelonephritis	72	2.05
M. H.	36	Obstructive uropathy	72	1.32
J. K.	38	Benign nephrosclerosis	73	1.80
J. Ki.	41	Benign nephrosclerosis	80	2.10
R. W.	35	Mitral stenosis; azotemia	84	2.17
O. R.	38	Acute glomerulonephritis, subsiding	93	1.91

*Receiving oral chlorothiazide 0.5 to 1.0 Gm. daily.

RESULTS

The results are summarized in tabular form in Table I. All values for filtration rate represent the means of at least 3 consecutive urine collection periods. Similarly, the serum magnesium concentrations represent the means of the determinations performed on the blood samples drawn at approximately the mid-point of each urine collection period. Without exception, the total serum magnesium concentration did not exceed the normal range of 1.7 to 2.2 mEq. per liter until the filtration rate was reduced to approximately 30

ml. per minute or below. At that point, with only one exception, the serum magnesium rose to well above the upper limits of normal as determined in our laboratory (2.2 mEq. per liter). The one patient whose serum magnesium remained within normal limits despite a reduction in filtration rate below 30 ml. per minute had been receiving daily doses of chlorothiazide prior to the study.

DISCUSSION

Renal mechanisms for the excretion of magnesium must play an important role in regulating the plasma magnesium level. It can be seen from the above data that the occurrence of hypermagnesemia in renal disease is closely related to the degree of reduction in filtration rate. When the filtration rate was reduced to (or below) 30 ml. per minute, all values for serum magnesium were consistently elevated with but one exception. Similar observations have been made in relation to the rise of serum phosphorus which occurs in renal failure.[11] Recently, Hammarsten and Smith[12] reported a positive correlation between the rise of serum magnesium and the increase in nonprotein nitrogen which occurs in patients with renal disease. Their data, as well as ours, would support the concept that one of the most important variables determining the renal excretion of magnesium is the glomerular filtration rate. In normal man, probably not more than 8 to 10 per cent of the filtered load of ionized magnesium is excreted in the urine per 24 hours. With a reduction in the filtered load of magnesium as in renal disease, less magnesium is available for excretion in the urine, and the serum concentration rises. This would be particularly true if either the tubular reabsorptive mechanisms remained intact or if passive back-diffusion occurred through damaged tubule cells. Since no partition of the total serum magnesium was done, it is impossible to state whether or not the increase of serum magnesium is primarily of the ionized component. Other data indicate that such is the case in uremia.[13] Excluding the possibility of the filtration of protein-bound magnesium through damaged glomeruli, this would seem a reasonable observation since a reduction in filtration rate is closely related to the rise of serum magnesium. The present data certainly provide no information as to whether or not tubular secretion of magnesium may occur, but would indicate that if such a mechanism does exist that it is quantitatively unable to maintain a normal serum, magnesium level in the presence of a fall in filtration rate to 30 ml. per minute.

Our observations also provide an explanation for the conflicting data in the literature as to whether the total serum concentration of magnesium is high, normal, or low, in patients with renal disease. Since quantitative changes in renal function studies had not been related to the serum magnesium concentration in these previously reported cases, it seems probable that the normal or low serum magnesium values were found in patients with filtration rates higher than 30 ml. per minute.

Under certain conditions, the serum magnesium may be normal despite a reduction in filtration rate to 30 ml. per minute or below. Our patient (S. M.) had been receiving 0.5 to 1.0 Gm. of oral chlorothiazide daily for 8 to 10 days

prior to the study period. There is some evidence that chlorothiazide may increase the urinary excretion of magnesium in normal man.[14] Such a drug action could be responsible for the normal serum magnesium in this patient. Unfortunately, careful studies as to the effect of chlorothiazide on the urinary excretion and serum concentration of magnesium were not feasible in this patient. Other drug and/or dietary factors which affect the serum level of magnesium must be considered in addition to the possible effects of chlorothiazide. It has been previously shown that the intravenous infusion of calcium salts in dogs results in an increase in the urinary excretion of magnesium.[15] Other data suggest that perhaps the fall of serum magnesium in patients with normal renal function following the intravenous administration of calcium gluconate is due to the movement of extracellular magnesium into the intracellular compartment or bone, and not due to an increase in the urinary excretion of magnesium.[16] It does not seem likely that patients with filtration rates of 30 ml. per minute, or lower, would ingest a diet containing such small amounts of magnesium as to result in depression of the serum value. On the other hand, conditions of magnesium loading might raise the serum level in patients with filtration rates higher than 30 ml. per minute. Excluding such drug or dietary factors, a rise in the total serum magnesium concentration in subjects with renal disease is related to the magnitude of fall in filtration rate.

SUMMARY

1. Simultaneous determinations of filtration rate and total serum magnesium concentration were done on 18 men with various types of renal disease.

2. With one exception, all patients with filtration rates less than 30 ml. per minute exhibited a distinct rise in the total serum concentration of magnesium above normal values.

3. One patient receiving chlorothiazide had a normal serum magnesium level in the presence of a fall in filtration rate to below 30 ml. per minute.

4. The hypermagnesemia of renal disease is closely related to the degree of reduction in glomerular filtration rate.

We would like to express our appreciation to Mrs. Eleanor Doyle, Mrs. Frances Rogers, and Mrs. Duncan Lewis for their technical assistance.

REFERENCES

1. Berliner, R. W.: Renal Excretion of Water, Sodium, Chloride, Potassium, Calcium and Magnesium, Am. J. Med. **9**: 541-559, 1950.
2. Hirschfelder, A. D.: Effect of Renal Insufficiency Upon Plasma Magnesium and Magnesium Excretion After Ingestion of Magnesium Sulfate, J. Biol. Chem. **104**: 647-653, 1934.
3. Heller, B. I., Hammarsten, J. F., and Stutzman, F. L.: Concerning Effects of Magnesium Sulfate on Renal Function, Electrolyte Excretion, and Clearance of Magnesium, J. Clin. Invest. **32**: 858-861, 1953.
4. Womersley, R. A.: Studies on the Renal Excretion of Magnesium and Other Electrolytes, Clin. Sc. **15**: 465-471, 1956.
5. Barker, E. S., Clark, J. K., and Elkinton, J. R.: Renal Excretion of Magnesium as Influenced by Lactate Administration and Certain Other Acute Experimental Condition, Federation Proc. **14**: 8, 1955.
6. Barker, E. S., Clark, J. K., and Elkinton, J. R.: Renal Response to Magnesium Loading in the Dog, Federation Proc. **16**: 6, 1957.

7. Hirschfelder, A. D.: Clinical Manifestations of High and Low Plasma Magnesium; Dangers of Epsom Salts Purgation in Nephritis, J. A. M. A. **102**: 1138-1141, 1934.
8. Walker, B. S., and Walker, E. W.: Normal Magnesium Metabolism and Its Significant Disturbances, J. Lab. & Clin. Med. **21**: 713-720, 1936.
9. Schreiner, G. E.: Determination of Inulin by Means of Resorcinol, Proc. Soc. Exper. Biol. & Med. **74**: 117-120, 1950.
10. Garner, R. J.: Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow, Biochem. J. **40**: 828-831, 1946.
11. Goldman, R., Bassett, S. H., and Duncan, G. B.: Phosphorus Excretion in Renal Failure, J. Clin. Invest. **33**: 1623-1628, 1954.
12. Hammarsten, J. F., and Smith, W. O.: Serum Magnesium in Renal Diseases, Clin. Res. **6**: 152, 1958.
13. Prasad, A. S., Flink, E. B., Zinneman, H. H., and McCollister, R.: Magnesium-protein Relationship and Status of Ultrafilterable Magnesium in Normal and Abnormal Human Sera, Clin. Res. **6**: 260-261, 1958.
14. Robinson, R. R., Murdaugh, H. V., Jr., and Peschel, E.: Unpublished observations.
15. Mendel, L. B., and Benedict, S. R.: The Paths of Excretion for Inorganic Compounds, Am. J. Physiol. **25**: 23-33, 1909.
16. Eliel, L. P., Smith, W. O., and Haurylko, J.: Metabolic Interrelations of Calcium and Magnesium in Patients With and Without Osteolytic Disease, J. Clin. Invest. **36**: 887, 1957.

STIMULATION OF GASTRIC PEPSIN SECRETION IN MAN BY HYPERVENTILATION AND BY DIAMOX

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IN ATTEMPTING to interfere with the carbonic anhydrase mechanism of gastric secretion[1, 2] in man, the effects of hyperventilation, to reduce the CO_2 supply, were contrasted to the effects of inhibition of the enzyme itself by Diamox. Both hyperventilation[5] and Diamox[3, 4] have been shown previously to reduce the secretion of hydrogen ions, but none of the reported studies have described any measurements of the effects on other constituents of gastric secretions. This study reports the effects of hyperventilation and of Diamox on H, Cl, Na, K, pepsin, and protein in the gastric juice. Both caused only modest decreases in acid secretion, but produced highly significant increases in pepsin secretion. The report deals with attempts, so far unsuccessful, to identify the mechanism of stimulation of pepsin secretion by either hyperventilation or by Diamox.

METHODS

Studies were performed on 14 fasting subjects, comprising 7 normal students, 2 vagotomized men with otherwise intact stomachs, and 5 patients with peptic ulceration (2 gastric and 3 duodenal). Experiments were done either in the basal state or during the intravenous infusion of 400 μg histamine base per hour, given as described elsewhere.[6] After a reasonably constant rate of secretion was found for 3 consecutive 15-minute periods, voluntary hyperventilation was performed for 15 or 30 minutes in 20 experiments. All subjects had tingling of the extremities at least and most exhibited Chvostek's sign; several also had carpopedal spasm.

Diamox (2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide) was injected intravenously in 5 subjects in doses of from 20 to 75 mg. per kilogram of body weight in 15 to 30 minutes; 3 were injected during histamine infusion and 2 while in the basal state.

Gastric juice was collected through a Levin tube by constant suction, supplemented at 15-minute intervals by hand suction with a syringe to complete consecutive 15-minute samples; these were assayed for free H^+ ion (end point pH 3.3), total chloride concentration, and pepsin concentration by methods previously described,[6] as well as for Na^+ and K^+ concentration by flame photometry; protein concentrations were measured with the biuret reagent. Forearm venous blood was collected without stasis in heparinized syringes and the pH measured immediately in 17 hyperventilation experiments.

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Supported by Grant R.G. 4298c from the United States Public Health Service.
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RESULTS

Hyperventilation.—

During histamine infusion: The effects of hyperventilation on the steady-state maximal secretion during histamine infusion was studied in 9 experiments on 7 subjects (Table I). Fifteen minutes of hyperventilation produced a highly significant prompt increase ($p < 0.005$) in concentration and output of pepsin and protein in the gastric juice, and a modest, although probably significant ($p < 0.05$) drop in H ion concentration. The output of Na^+ increased but this was probably not statistically significant ($p = 0.05$). These effects persisted for 15 minutes after the end of the hyperventilation. No significant effects on volume, on chloride, or potassium were observed. Volume increased in 2 or 3 subjects, but identical changes in composition of the juice occurred and were as marked in the others who had no changes in volume secretion.

TABLE I. EFFECT OF HYPERVENTILATION FOR 15 MINUTES DURING CONTINUOUS HISTAMINE INFUSION ON VOLUME AND COMPOSITION OF GASTRIC JUICE IN 9 STUDIES (MEANS ± S.E.M.)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	−30	57.4 ± 9.9	1,410 ± 201	310 ± 25	117 ± 5.9	13 ± 2.3	18.1 ± 0.6	156 ± 3.3
	−15	50.0 ± 5.0	1,223 ± 135	310 ± 40	127 ± 5.0	12 ± 2.4	18.0 ±0.7	162 ± 4.4
Hyperventilation	15	66.2 ± 9.3	2,162* ± 240	534† ± 48	112 ± 4.5‡	16 ± 1.9	18 ± 0.9	155 ± 3.6
Recovery	30	56 ± 5.6	1,765‡ ± 204	444‡ ± 63	113 ± 6.3‡	16 ± 2.1	17.5 ± 0.9	156 ± 4.0
	45	55 ± 6.2	1,390 ± 171	398 ± 74	121 ± 3.7	15 ± 2.2	16.8 ± 0.7	157 ± 3.6

P.U. = pepsin units.
*$P<0.005$; by "t" test significance of difference of mean from mean control −15 minutes.
†$P<0.001$.
‡$P<0.05$.

TABLE II. EFFECT OF 30-MINUTE HYPERVENTILATION DURING BASAL SECRETION ON VOLUME AND COMPOSITION OF GASTRIC JUICE IN 2 SUBJECTS (MEAN VALUES)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	−30	28	1,150	750	39	58	15.0	122
	−15	28	1,080	750	43	58	14.0	130
Hyperventilation	15	45	2,255	620	45	62	14.0	129
	30	30	2,560	1,030	62	44	15.5	135
Recovery	45	46	1,675	880	68	44	14.8	138
	60	26	1,480	525	84	31	14.4	143

In the basal state (Table II): In 2 subjects, hyperventilation for 30 minutes produced the same striking pepsin increases during the first 15 minutes in the basal state as during histamine infusion. The H^+ concentration, however, did not fall and in fact started rising during the second 15-minute period

and continued to increase after that, accompanied by an increase in chloride concentration and a concomitant fall in sodium concentration. Potassium was unaffected. The stimulation of pepsin secretion was therefore quicker and out of proportion to the stimulation of acid secretion.

Thirty-minute Hyperventilation.—Two successive 15-minute periods of hyperventilation were performed by 5 subjects, 3 during histamine and 2 in the basal state. In one basal and one histamine study, the plateau of pepsin concentration was reached in the first period; in the other 3, the pepsin concentration continued to rise in the second period and in 3 subjects reached levels not seen even in hypoglycemic or any other type of stimulation. For the 5 subjects the mean concentrations for 6 successive 15-minute periods (2 control, 2 hyperventilation, and 2 after) were 1,630, 1,400, *2,682, 3,250,* 2,062, and 1,572 pepsin units per milliliter, respectively.

Other experiments were done to investigate possible mechanisms whereby hyperventilation might produce these effects on pepsin and protein secretion.

Cholinergic Pathways.—

Central cholinergic (vagal): Two patients, who had been vagotomized 1 and 5 years before, respectively, and who had intact stomachs were studied. The completeness of vagal section in both was confirmed by insulin hypoglycemia, which in neither produced any stimulation of gastric secretion within an hour of adequate hypoglycemia (true blood glucose less than 40 mg. per cent).

TABLE III. EFFECT OF 15-MINUTE HYPERVENTILATION DURING HISTAMINE INFUSION IN VAGOTOMIZED SUBJECTS (MEAN OF 3 EXPERIMENTS)
(NOTE: DELAYED INCREASE IN PEPSIN CONCENTRATION)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	–30	22	1,570	–	62	36	16.7	123
	–15	20	1,432	–	62	30	16.7	115
Hyperventilation	15	31	1,667	–	67	35	14.1	123
Recovery	30	21	2,187	–	64	44	13.3	128
	45	28	1,350	–	67	42	12.5	125

Three hyperventilation studies were carried out during subsequent histamine infusion (Table III) and in both subjects a well-marked increase in pepsin secretion was noted. The response differed somewhat from the normal subjects in that the acid concentration did not fall and the maximal concentrations of pepsin were found in the 15-minute period after hyperventilation. In no instance was this the case in the intact subjects where the maximal increases always occurred during the hyperventilation.

Peripheral cholinergic mechanism: In 3 subjects with intact stomachs a dose of atropine was determined which would prevent hypoglycemic stimulation of gastric secretion (between 1.0 and 1.5 mg. of atropine sulfate intravenously). These doses of atropine were given during subsequent histamine

infusions and as will be seen by comparing Tables I and IV, atropine under these conditions decreased volume as well as the pepsin, protein, and potassium concentrations. These effects of atropine last for at least an hour. Hyperventilation during these 3 studies produced a fall in H^+ concentration and a rise in sodium, pepsin, and protein concentrations (Table IV) as was the case without atropine (Table I). The increase in pepsin and protein persisted at a high level in the postventilation period, thus resembling the response in the vagotomized subjects.

TABLE IV. EFFECT OF 15-MINUTE HYPERVENTILATION OF 3 ATROPINIZED SUBJECTS DURING HISTAMINE INFUSION (MEANS)

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	–30	49	780	195	123	16.7	12.7	155
	–15	29	630	190	125	15.3	12.0	152
Hyperventilation	15	36	1,373	410	109	21.3	11.8	150
Recovery	30	42	1,307	390	104	31.3	12.8	153

Noncholinergic Mechanisms.—

Possible deionization of calcium: The possibility of preventing the effects of hyperventilation by concomitant calcium administration was studied in two subjects, who were hyperventilated on 3 occasions during histamine infusion and were found to give the typical normal response as seen in Table I. During a subsequent period of hyperventilation in the same study, 30 ml. of 10 per cent calcium gluconate was injected intravenously. One subject had tetany during hyperventilation whether calcium was injected or not; the other had tetany without calcium but no tetany with calcium. Pepsin secretion increased to an extent not significantly different from that seen without calcium injections.

Changes in Blood pH.—

By hyperventilation: Following hyperventilation the pH of the blood rose by 0.07 to 0.30 pH (mean 0.156) unit in all experiments except in one, where the level remained unchanged at pH 7.68. There was no correlation between blood pH and the concentration of pepsin in the gastric juice ($r = +0.26$) (Fig. 1), both apparently increasing independently of the other during hyperventilation. It will be seen that even before hyperventilation the blood pH was above normal, the result of the removal of secreted gastric acid. The increase in pH with hyperventilation was not correlated with the fall in H+ concentration which occurred.

By infusion of bicarbonate (Table V): In 3 of the subjects in whom hyperventilation had produced typical changes in gastric juice composition, an attempt was made to reproduce the alkalosis by rapidly infusing intravenously 5.6 per cent sodium bicarbonate, during established I.V. histamine

stimulation. The 3 subjects received 180, 200, and 350 ml. of this solution (119, 133, and 233 mEq. of base), respectively, in 15 minutes. In each, the blood pH rose by 0.1 pH unit or more, and the first had tetany. None of the effects of hyperventilation on gastric secretion were reproduced by the bicarbonate alkalosis. A significant ($p < 0.005$) progressive drop in potassium concentration in all 3 subjects was the only gastric effect noted.

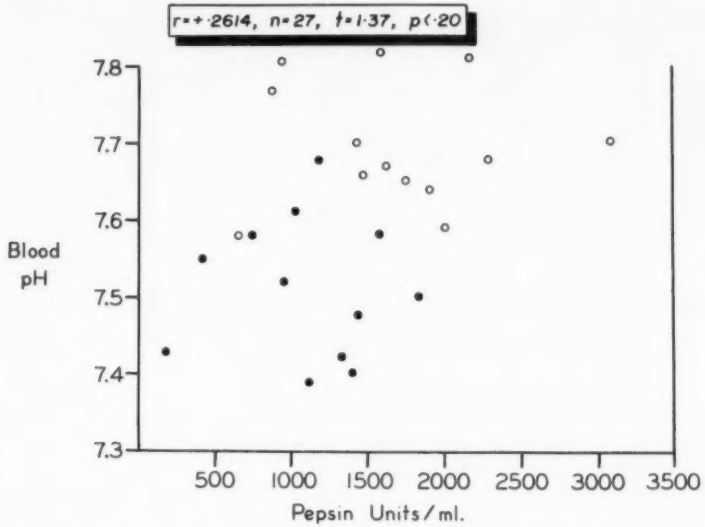

Fig. 1.—Blood pH and pepsin output before (●) and after (○) hyperventilation showing lack of correlation.

TABLE V. EFFECT OF INTRAVENOUS INFUSION OF 5.6 PER CENT $NaHCO_3$ DURING CONTINUOUS HISTAMINE INFUSION IN 3 SUBJECTS

PERIOD	MINUTES	VOLUME (ML./15 MIN.)	P.U./ML.	PROTEIN (MG. %)	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Control	–45	68	1,270	370	122	16	19.3	158
	–30	78	1,603	450	125	14	20.0	160
	–15	68	1,270	330	116	20	17.7	158
			Bicarbonate Infusion in 15 Minutes					
	15	56	1,340	260	113	21	16.6	152
	30	65	1,523	320	123	21	15.3	154
	45	60	1,510	265	126	15	16.0	157
	60	48	1,370	320	132	11	15.7	157

Plasma Pepsinogen.—As an incidental observation in 2 subjects in whom an adequate pepsin-protein response to hyperventilation was observed, no change occurred in the concentration of pepsinogen in the plasma. It was not measured in the other subjects. This suggests that the peptic cell does not develop a nonspecific increase in permeability to protein as a result of hyperventilation.

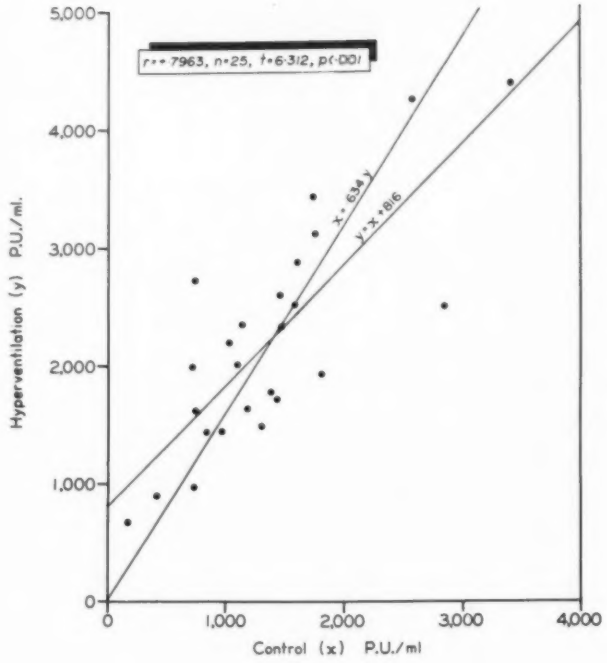

Fig. 2.

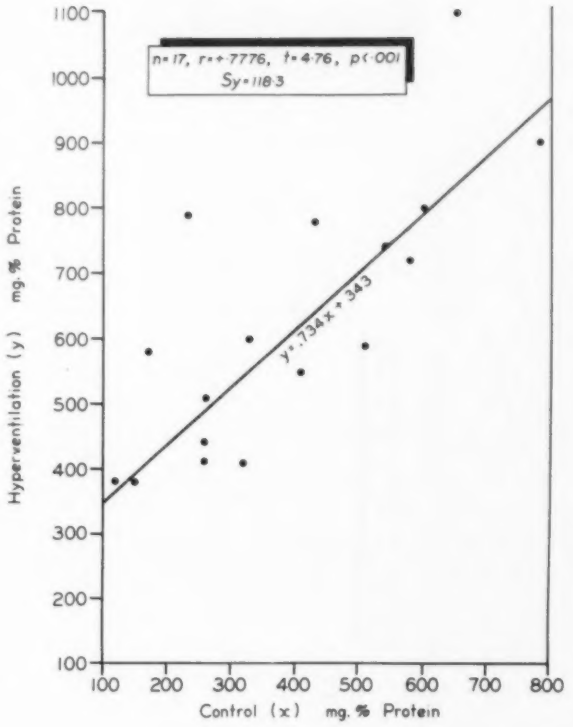

Fig. 3.

Fig. 2.—Significant positive linear correlation between gastric juice pepsin concentrations before and during hyperventilation.
Fig. 3.—Positive linear correlation between gastric juice protein concentrations before and during hyperventilation.

The nature of the pepsin increase: Fig. 2 shows the concentration of pepsin during hyperventilation in all the studies, plotted against the concentration in the preceding 15-minute period. It includes the five 30-minute studies in which the pepsin concentration in the second 15-minute period is plotted against that in the first. The correlation is positive, linear, and highly significant ($r = +0.796$, $p < 0.001$). A similar significant ($r = +0.792$) correlation was found between the *output* of pepsin before and during hyperventilation. In 17 studies where the protein concentration was measured, a similar positive linear correlation was found (Fig. 3) ($r = +0.776$, $p < 0.001$).

The increase in protein is probably accounted for by the increase in pepsin concentration, as suggested by the mean concentration of pepsin per milligram of protein. These are 4.54, 3.95 (hyperventilation), 4.04, 3.98, and 3.49 protein units per milligram for the respective 15-minute periods in the histamine studies. In the individual cases, however, the ratio of pepsin to protein is not as constant as it is for the whole group. No attempt has been made to determine more exactly the specific potential peptic activity of the protein in the gastric juice.

TABLE VI. EFFECTS OF DIAMOX DURING CONTINUOUS HISTAMINE INFUSION ON GASTRIC JUICE VOLUME AND COMPOSITION (MEANS ± S.E.M.)

PERIOD	VOLUME (ML./30 MIN.)	P.U./ML.	H^+ (mEq./L.)	Na^+ (mEq./L.)	K^+ (mEq./L.)	Cl^- (mEq./L.)
Mean 2-hour control (n = 20)	81.3 ± 11.8	1,132 ± 74	75.9 ± 7.3	24.0 ±2.4	16.57 ± 0.34	127 ± 7
Diamox Intravenous						
Post-Diamox 30 minutes (n = 10)*	82.2 ± 19.9	1,233 ± 82	80.3 ± 11.7	24.2 ± 2.0	14.2 ± 0.48	126 ± 10
Post-Diamox 60 minutes	65.6 ± 11.6	1,477 ± 87	72.5 ± 10.3	31.1 ± 2.7	13.0 ± 0.49	125 ± 9
Post-Diamox 90 minutes	64.8 ± 15.1	1,552 ± 128	55.9 ± 11.8	41.3 ± 3.5	11.8 ± 0.42	128 ± 7
Post-Diamox 120 minutes	66.0 ± 14.1	1,667 ± 109	63.3 ± 12.6	44.1 ± 7.2	11.3 ± 0.25	128 ± 6

*n = Number of samples.

The Effects of Diamox.—Diamox in a total dosage of 1.2 to 3.75 Gm. (20 to 75 mg. per kilogram of body weight) was given intravenously to 3 subjects during histamine infusion and to 2 others during basal secretion. The effects were essentially similar and are combined in Table VI. The reduction in both volume and in acid concentration, each not quite significant statistically, resulted in a marked reduction in the calculated output of acid following the injection of Diamox. The chloride concentration, however, remained unchanged; the fall in potassium concentration was highly significant as was the rise in sodium concentration. The sum of cations, like the concentration of chloride remains unchanged after Diamox. The concentration of pepsin increased significantly and continued to rise steadily up to 2 to 3 hours after the Diamox injection.

DISCUSSION

With the present state of knowledge of pepsinogen secretion[10] it is not possible to interpret the gastric juice pepsin increases following hyperventilation or Diamox in terms of mechanisms.

It would seem probably that hyperventilation exerts its effect on pepsin secretion neither through the vagus nor through local cholinergic mechanisms, and, furthermore, that the effects are probably not due to an alteration of blood pH per se nor to altered cell permeability to pepsinogen. The role of calcium deionization has not been fully evaluated if, in fact, such deionization occurs with hyperventilation.[8] The possible role of increased gastric motility, which occurs after hyperventilation,[9] has not been evaluated, but it is difficult to see how such changes in motility could be concerned with an increase in pepsin secretion. Furthermore, the persistence of increased pepsin and protein secretion suggests that the mechanical act of hyperventilation as such has no direct effect on pepsin secretion. Whatever the mechanisms, however, they appear to act independently of the known pathways of stimulation of pepsin secretion in man, where both acid and pepsin secretion are simultaneously stimulated[10] by the known stimulants.

The changes in hydrogen ion which occurred with both hyperventilation and with Diamox might be interpreted in terms of the carbonic anhydrase mechanisms thought to be responsible for the supply of hydrogen ions to the secreting mucosa. These occurred without significant changes in chloride concentrations, and are similar to the dissociation of H and Cl which occurs when an electric current is passed from mucosa to serosa, opposite in direction to the normal mucosal electromotive force.[7]

SUMMARY

In 25 studies on 14 subjects voluntary hyperventilation for 15 to 30 minutes invariably caused a very significant increase in the concentration and output of pepsin in the gastric juice. This occurred whether hyperventilation was performed during continuous histamine infusion, in the basal state, following complete vagotomy, or during adequate atropinization. It could not be reproduced by equivalent alkalosis produced by intravenous $NaHCO_3$ infusion. The effect persisted for 15 minutes after the cessation of hyperventilation. The mechanisms of this response have not been elucidated and appear to involve none of the known pathways for stimulation of pepsin secretion. Diamox given intravenously also increased the secretion of pepsin.

The effects of hyperventilation and of Diamox on gastric juice volume and hydrogen ion, sodium, potassium, chloride, and protein concentrations reported in the paper shed no further light on the mechanisms of pepsin stimulation.

REFERENCES

1. Davenport, H. W.: Gastric Carbonic Anhydrase, J. Physiol. **97**: 32, 1939.
2. Davies, R. E.: Gastric Hydrochloric Acid Production—The Present Position, *in* Metabolic Aspects of Transport Across Cell Membranes edited by Q. R. Murphy, Madison, 1957, The University of Wisconsin Press, Chap. XII.
3. Davies, R. E., and Roughton, F. J. W.: Hydrochloric Acid Production by Isolated Gastric Mucosa, Biochem. J. **42**: 609, 1948.
4. Janowitz, H. D., H. Colcher, and F. Hollander: Inhibition of Gastric Secretion of Acid in Dogs by Carbonic Anhydrase Inhibitor, 2-Acetyl Amino-1, 3, 4-Thiadiazole-5-Sulfonamide, Am. J. Physiol. **171**: 325, 1952.

5. Browne, J. S. L., and Vineberg, A. M.: The Interdependence of Gastric Secretion and the CO_2 Content of the Blood, J. Physiol. **75**: 345, 1932.
6. Hirschowitz, B. I., London, J. A., and Wiggins, H. S.: Differential Diagnosis of Gastric Ulcer and Cancer by a Study of Maximally Stimulated Gastric Secretion, J. LAB. & Clin. MED. **50**: 447, 1957.
7. Rehm, W. S., and Dennis, W. H.: A Discussion of Theories of Hydrochloric Acid Formation in the Light of Electrophysiological Findings, *in* Metabolic Aspects of Transport Across Cell Membranes edited by Q. R. Murphy, Madison, 1957, The University of Wisconsin Press, Chap. XIV.
8. Brown, E. B. J.: Physiological Effects of Hyperventilation, Physiol. Rev. **33**: 445, 1953.
9. Danielopolu, D., and Savulesco, A.: Effects de l'hyperventilation sur la motilite gastrique, J. physiol. et path. gén. **26**: 225, 1928.
10. Hirschowitz, B. I.: Pepsinogen: Its Origins, Secretion and Excretion, Physiol. Rev. **37**: 475, 1957.

COMPARATIVE TOLERANCE TO POISONS OF NORMAL LIVER TISSUE AND REGENERATED LIVER TISSUE AFTER MAJOR HEPATECTOMY

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AFTER the surgical removal of 70 per cent or more of the liver in human beings and dogs, sustenance of life is dependent on the ability of the normal residual remnant plus its regenerated component to carry out the vital functions commonly attributed to this organ. Its ability to do so has been proved by clinical experience and experimental laboratory investigations. The entire right lobe of the human liver is being resected with increasing frequency for such indications as primary malignant tumors (hepatoma, cholangiocarcinoma, and somatic sarcoma, unilobar metastases, cancer of the gall bladder, and occasional benign tumors. The right lobe consists of 80 per cent of the weight of the human liver, yet it now is feasible and relatively safe to perform total right hepatic lobectomy by the controlled hemostatic procedure of preliminary ligation and severance of the right hepatic duct, the right hepatic artery, and the right branch of the portal vein prior to transection of the interlobar septum. A residual 20 per cent of the liver substance is enough for the maintenance of life if the subject is an otherwise normally functioning organism. The tested liver functions are minimally disturbed by the removal of the human right hepatic lobe except for transient hypoproteinemia.

In our experiments, the rapidity and completeness of liver regeneration after subtotal hepatectomy appear to be inversely proportional to the size of the animal. In rats, the liver has completely regenerated within 12 to 18 days after major lobectomies. In dogs the remnant of residual liver after 70 per cent hepatectomy has grown to the original proportion within 8 weeks after the operation. In the human being the process of regenerative hyperplasia is not so easily followed because of the infrequency of "second-look" observations and measurements, but we found in two patients, on exploratory

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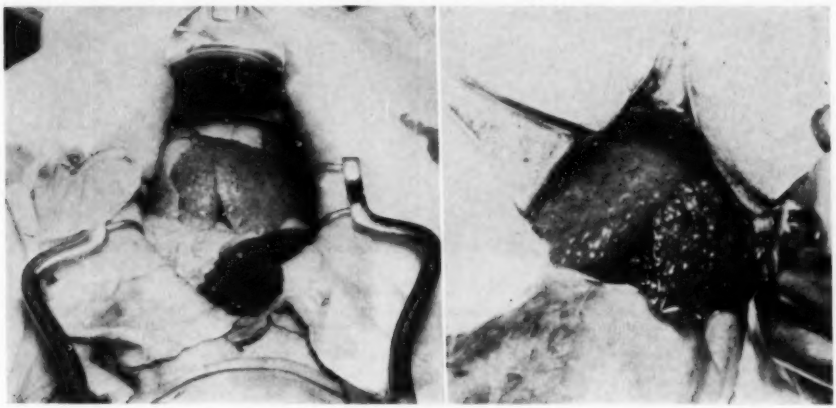
This project was supported in part by a field investigation grant (C-2027) from the National Cancer Institute, National Institutes of Health, United States Public Health Service, Washington, D. C.

Received for publication Aug. 25, 1958.

laparotomy 2 years after total right hepatic lobectomy, that the left lobe had expanded to fill the entire cupola of the diaphragm, assuming final dimensions as great, or greater, than the original liver.

The minimal deviations from normal in the numerous functions of the liver after total right hepatic lobectomy have been a source of security for the patient and of assurance to the surgeon. Not so much is known of the regenerated liver's tolerance or adaptation to unusual or "stress" conditions to which even the normal intact liver is not ordinarily subjected. One such function is the detoxification of poisons. We thought it of interest to see if the regenerated liver of dogs possessed the ability to detoxicate carbon tetrachloride to the same degree as the normal intact liver of other dogs.

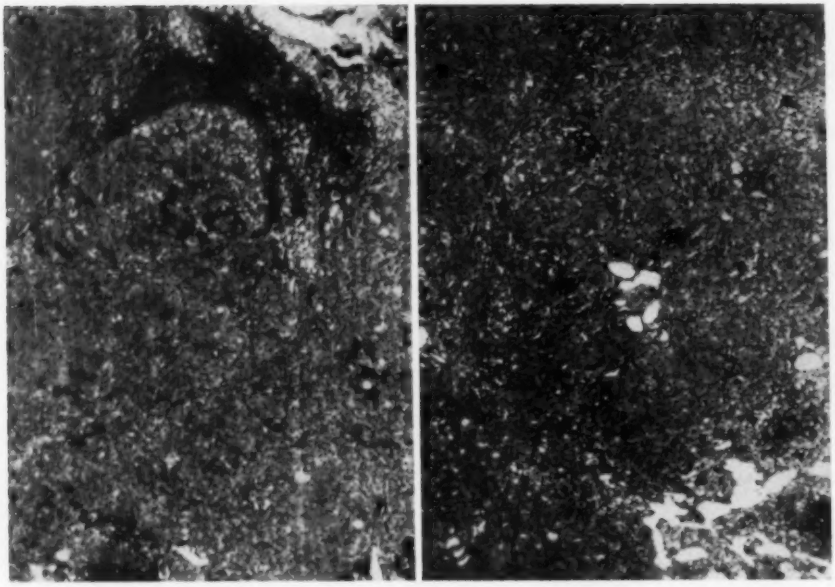


A. *B.*

Fig. 1.—*A*, Experimental cirrhosis in dog with intact liver. View at laparotomy of an adult mongrel dog which received 270 ml. of carbon tetrachloride over a period of 9 months. The liver appears coarsely nodular with a uniform but distributed mottled appearance involving the entire capsular surface. The nodules range in size downward from 1 cm. in diameter. The liver is pale in contrast to the deep red spleen. *B*, Experimental cirrhosis in dog after major hepatectomy. View at laparotomy of the liver of a dog which received carbon tetrachloride during a 9 months' course. The exhibition of the drug was initiated 4 months after a 70 per cent partial hepatectomy. The 70 per cent partial hepatectomy was performed 2 months after a previous 50 per cent hepatectomy. The liver surface was nodular and tan with some patches of reddish hue.

METHODS

Carbon tetrachloride was used as the toxic agent. Dogs of equal weight were used for this study. Some dogs had been operated on two months before the experiment started and had 70 per cent of their livers removed. The control dogs had no operation and therefore their livers were intact. At the beginning of the experiment these dogs were weighed and carbon tetrachloride was inserted through an isolated loop of ileum. Three milliliters of carbon tetrachloride was introduced every other day for 3 months. Then enteric capsules containing 5 ml. of carbon tetrachloride were given by mouth to these dogs every other day for 6 months. All dogs were weighed weekly and liver function tests were done each month. No special diet was given. At the end of the experiment all dogs were surgically explored. The peritoneal cavity contained 600 to 1,200 ml. of clear, yellowish fluid. The livers of both groups of animals were granular, hard, and yellowish. Small segments of these cirrhotic livers were removed for histologic study.



A. *B.*

Fig. 2.—*A*, Liver of an adult mongrel dog which had received 270 ml. of carbon tetrachloride over a period of 9 months, starting 4 months after a 70 per cent hepatectomy. The 70 per cent partial hepatectomy was performed 2 months after a 50 per cent partial hepatectomy. This low-power microphotograph shows extensive fresh hemorrhage in regions of necrosis. Significant remnants of residual normal hepatic architecture cannot be identified in this section. The sinusoidal patterns are absent. *B*, Same liver as in *A*. The liver in this biopsy shows acute massive necrosis. In the center of the microphotograph are intact surviving parenchymal cells, surrounding vascular channels. There are foci of fresh hemorrhage infiltrating the necrotic parenchyma. The sinusoidal channels are not patent.

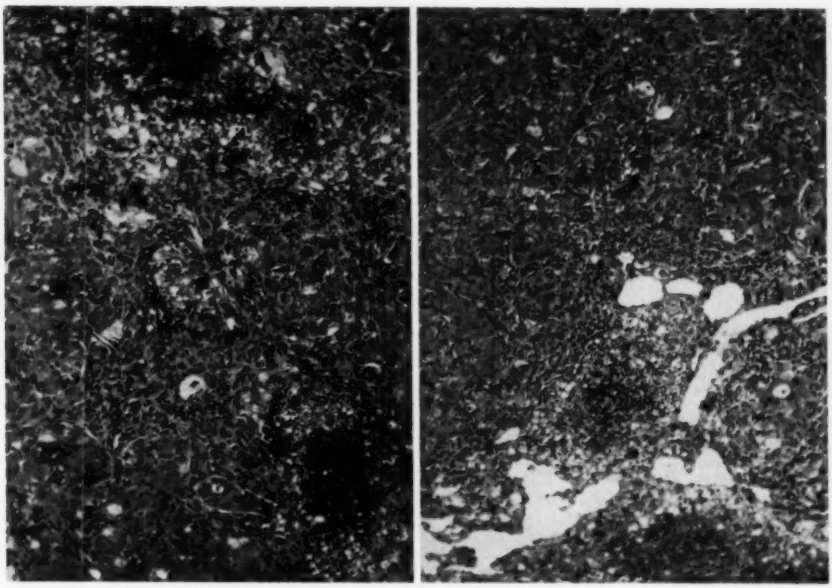
RESULTS

1. The alterations of liver function tests in those dogs with regenerated livers and the other dogs without partial hepatectomy were essentially the same.

2. The transient hypoproteinemia (hypoalbuminemia and hypoglobulinemia) associated with the toxic necrosis was considerably less and was restored earlier in the dogs with regenerative hyperplastic livers following major hepatectomy than in the control dogs with intact livers.

3. The weight losses in both groups were somewhat similar, but 7 months from the date the experiment started both groups began to gain weight, which was due to ascites as a result of hepatic cirrhosis.

4. In the dogs which had no partial hepatectomy prior to the administration of carbon tetrachloride, more fluid was noted in the peritoneal cavity and the liver was firmer and more granular (Fig. 1, *A* and *B*). The histologic evidence of the toxic necrosis was of apparently equal degree in both groups of animals. (See Fig. 2, *A*, *B*, *C*, and *D*.)



C. *D.*

Fig. 2.—Cont'd. *C*, Biopsy of same liver as in *A* and *B*. Intermediate magnification. The residual viable parenchymal cells show no evidence of glycogen and normal sinusoidal patterns. Two large foci of hemorrhage into regions of necrosis. *D*, Biopsy of same liver as in *A*, *B*, and *C*. Extensive necrosis seen in the lower half of the microscopic field. The parenchymal cells are not oriented in the normal array of plates. There is no visible evidence of intracellular glycogen storage.

CONCLUSION

According to our investigation, the regenerated liver of the dog tolerates poison (specifically, carbon tetrachloride) as well as normal, intact liver, if not more so. The reason for this relative tolerance may be the fact that 2 months or more after major hepatectomy in the dog, the regenerated liver is larger and weighs more than the original liver. Its size represents true regenerative hyperplasia as we have shown by (1) chemical analyses for lipids and nitrogen of aliquot parts of the liver, (2) nuclear counts, and (3) comparable histologic structure as witnessed on microscopic study.

REFERENCES

1. Aterman, K.: Studies in Fibrosis of the Liver in the Use of Carbon Tetrachloride. 1. Relation Between Hepatocellular Injury and New Formation of Fibrous Tissue, A. M. A. Arch. Path. 57: 1-25, 1954.
2. Cameron, G. R., and Karunaratne, W. A. E.: Carbon Tetrachloride Cirrhosis in Relation to Liver Regeneration, J. Path. & Bact. 42: 1-21, 1936.
3. Edwards, J. E., and Dalton, A. J.: Induction of Cirrhosis of the Liver and of Hepatomas in Mice With Carbon Tetrachloride, J. Nat. Cancer Inst. 3: 19-41, 1942.
4. Gardner, G. H., Grove, R. C., Gustafson, R. K., Maire, E. D., Thompson, M. J., Wells, H. S., and Lamson, P. D.: Studies on the Pathological Histology of Experimental Carbon Tetrachloride Poisoning, Bull. Johns Hopkins Hosp. 36: 107-133, 1925.

5. Glynn, L. E., and Himsworth, H. P.: The Intralobular Circulation in Acute Liver Injury by Carbon Tetrachloride, Clin. Sc. **6**: 235-245, 1948.
6. Islami, A. H., Pack, G. T., and Hubbard, J. C.: Regenerative Hyperplasia of the Cirrhotic Liver Following Partial Hepatectomy, Cancer **11**: 663-686, July-Aug. 1958.
7. Lacquet, A. M.: Experimental Pathology of the Liver. VIII. Effects of Carbon Tetrachloride on the Normal and on the Restored Liver After Partial Hepatectomy, Arch. Path. **14**: 164-176, 1932.
8. Lamson, P. D., and Wing, R.: Early Cirrhosis of the Liver Produced in Dogs by Carbon Tetrachloride, J. Pharmacol. & Exper. Ther. **29**: 191-202, 1926.
9. Moon, V. H.: Experimental Cirrhosis in Relation to Human Cirrhosis, Arch. Path. **18**: 381-424, 1934.
10. Moschcowitz, E.: Laennec Cirrhosis; Its Histogenesis, With Special Reference to the Role of Angiogenesis, Arch. Path. **45**: 187-215, 1948.
11. Pack, G. T., and Baker, H. W.: Total Right Hepatic Lobectomy. Report of a case, Ann. Surg. **133**: 253-258, 1953.
12. Pack, G. T., and Brasfield, R. D.: Metastatic Cancer of the Liver. The Clinical Problem and Its Management, Am. J. Surg. **90**: 704-716, 1955.
13. Pack, G. T., and Islami, A. H.: The Epiphenomenon of Total Right Hepatic Lobectomy, Surgery **40**: 611-614, 1956.
14. Pack, G. T., Miller, T. R., and Brasfield, R. D.: Total Right Hepatic Lobectomy for Cancer of the Gallbladder. Report of three cases, Ann. Surg. **142**: 6-16, 1955.
15. Stowell, R. E., and Lee, C. S.: Histochemical Studies of Mouse Liver After Single Feeding of Carbon Tetrachloride, Arch. Path. **50**: 519-537, 1950.
16. White, E. G.: The Effect of Carbon Tetrachloride on the Liver of the Pig, With Special Reference to Experimental Cirrhosis, J. Path. & Bact. **49**: 95-103, 1939.

LETHAL IRON OVERLOAD IN DOGS

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THE harmful effects of large tissue iron deposits have received much attention in recent years. In a previous study from this laboratory,[1] dogs which had received intravenously 0.5 to 1.0 Gm. of iron per kilogram of body weight lived for 4 to 7 years without developing the characteristic lesions of hemochromatosis in man. The purpose of this paper is to describe the effects in dogs of iron injected parenterally in regular doses until toxic manifestations appeared. The animals were then observed until death occurred or seemed imminent and the tissue changes at autopsy were recorded. One dog received saccharated iron oxide intravenously; 2 received iron-dextran intramuscularly; and 2 received iron-dextran intraperitoneally. The accumulation of 2.5 to 3.3 Gm. of iron per kilogram of body weight produced death within 5 to 10 months. The tissues showed massive hemosiderin deposits—chiefly in cells of the reticuluoendothelial system—with no fibrous tissue reaction. All the animals showed anorexia, weight loss, and a decrease in serum albumin concentration; one developed ascites, anasarca, and chronic leg ulcers. None of the dogs showed any evidence of diabetes or cirrhosis by laboratory or postmortem examinations. The exact cause of death and its relationship to the iron overload were not established.

MATERIAL AND METHODS

Two adult mongrel dogs were given injections of the iron-dextran solution (Imferon†) intramuscularly into alternate thighs in a dose of 2½ or 5 ml. (125 or 250 mg. elemental iron) per day 6 days a week. The schedule and duration of injections are shown in Fig. 1. Dog 1 received a total of 23 Gm. of iron (3.1 Gm./Kg.), and Dog 2 received 25 Gm. (2.5 Gm./Kg.) during a 5-month period.

Two littermate puppies (approximately 4 months old) were given injections of Imferon intraperitoneally into the left lower quadrant of the abdomen. The dosage schedule was 125 mg. of iron twice weekly for 3 weeks, then 250 mg. twice weekly for 19 weeks, and finally 250 mg. daily for 6 days in one puppy (Dog 4) and 500 mg. daily for 12 days in the other puppy (Dog 3). The total amount of iron injected was 9.75 Gm. (3.3 Gm./Kg.) and 14 Gm. (2.5 Gm./Kg.), respectively (Fig. 2).

Another adult mongrel dog was given injections of saccharated iron oxide (Ferrivenin†) intravenously in doses of 10 ml. (200 mg. of iron) per day 5 days a week for 28 weeks. A total dose of 29 Gm. of iron (3.0 Gm./Kg.) was given to this dog.

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This investigation was supported in part by Research Grant H-22 from the National Heart Institute, United States Public Health Service.

Received for publication Aug. 27, 1958.

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**Dr. Smith is now Professor of Pathology at the University of Virginia School of Medicine.

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The animals were housed in individual cages and were fed commercial dog chow. Hexylresorcinol as a vermifuge was given to each animal during an initial period of observation. Feces were tested for occult blood frequently, and the vermifuge treatment was repeated whenever blood was detected. The dogs were weighed at frequent intervals.

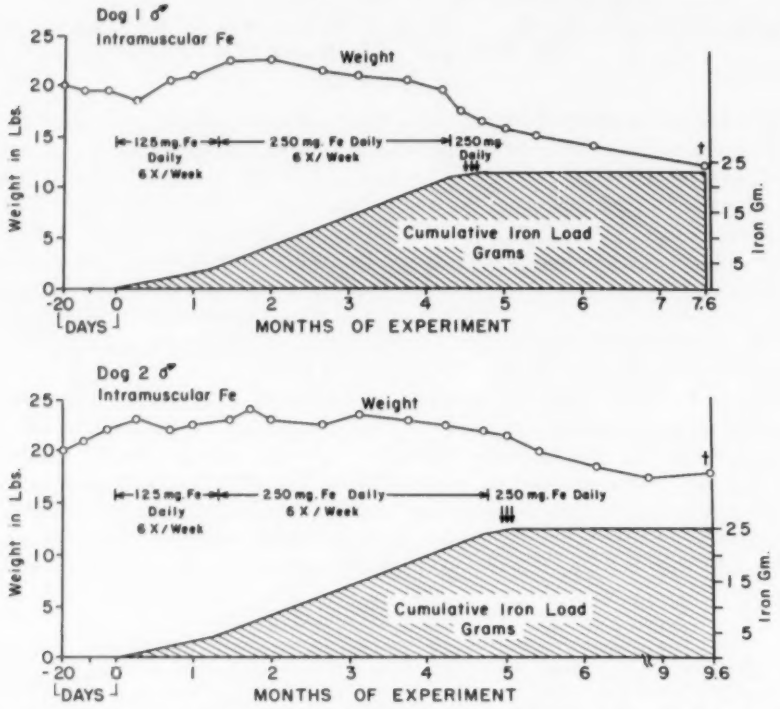

Fig. 1.—Correlation of body weight with cumulative iron load in dogs receiving intramuscular iron-dextran injections.

Periodic determinations of red cell counts, hemoglobin concentration, packed cell volumes, white cell counts, reticulocytes, serum iron, and unsaturated iron binding capacity were made by methods previously described.[1] In addition, measurements of fasting blood sugar, serum bilirubin, Bromsulphalein retention and serum protein fractions by chemical and paper electrophoretic techniques were performed at intervals on the dogs which received iron-dextran. At death, tissue iron estimations were made as previously described.[1] Histologic examination was performed on tissues fixed in neutral formalin and Zenker's acetic solutions. Sections were stained routinely with hematoxylin and eosin and for iron with a modified Gomori stain counterstained with Mallory's basic fuchsin.

RESULTS

A. Iron-Dextran Iron Overload.—

Local effects: Intramuscular injections of iron-dextran were given into the outer portion of the thighs without producing any tenderness or evidence

of tissue inflammation. The intraperitoneal injections of Imferon produced no discomfort or evidence of peritoneal irritation.

Generalized effects: No immediate or delayed generalized reactions to injections of iron-dextran occurred. Both adult dogs that received intramuscular injections maintained their weight throughout most of the injection period (Fig. 1). After approximately 4 months Dog 1 refused to eat, lost weight, and had occasional diarrhea, with benzidine-positive feces. These symptoms continued until he died 2½ months later. Anorexia and weight loss were less marked in Dog. 2. Nine days before death he developed complete anuria followed by total refusal of food, tarry diarrhea, and tense abdominal distention. When death seemed imminent, the animal was killed.

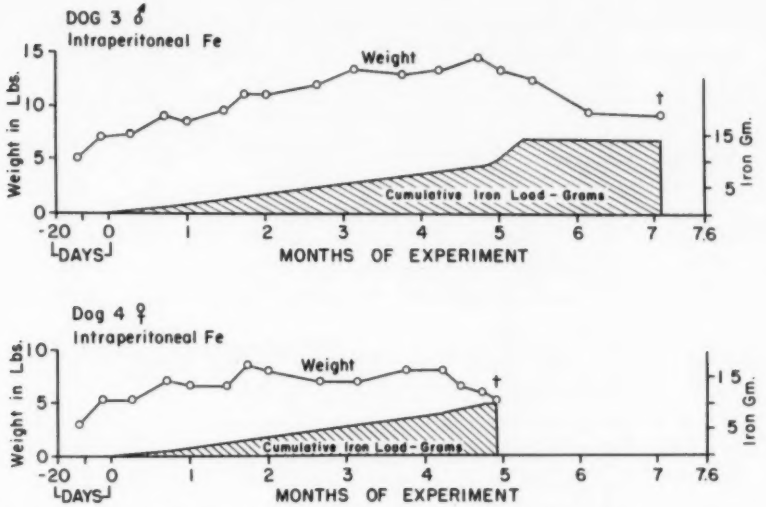

Fig. 2.—Correlation of body weight with cumulative iron load in dogs receiving intraperitoneal iron-dextran injections.

One puppy (Dog 3) that received intraperitoneal injections of iron-dextran doubled his weight during the injection period. However, during the last 2 weeks of injections he lost 2 pounds and continued to lose weight until he died (2 months later). Growth during the injection period was less pronounced in the other puppy (Dog 4) (Fig. 2). Her weight fell abruptly during the final period of intensive injections and she died soon afterward. Throughout the experiment both puppies remained vigorous. Mange occurred in each dog; it was mild in Dog 3, but extensive and unresponsive to treatment in Dog 4. There was no premonition of death in either animal.

Total survival time for Dogs 1 and 2 which had received their iron-dextran intramuscularly was 227 and 291 days, respectively. Puppies 3 and 4 survived 213 and 148 days from the beginning of their course of intraperitoneal iron-dextran injections.

TABLE I. HEMATOLOGIC VALUES OF DOGS RECEIVING IRON-DEXTRAN INJECTIONS

DAY OF EXP'T	R.B.C. (X 10^6)	HGB. (GM./100 ML.)	HCT. (%)	RETIC. (%)	W.B.C. (X 10^3)	DAY OF EXP'T	R.B.C. (X 10^6)	HGB. (GM./100 ML.)	HCT. (%)	RETIC. (%)	W.B.C. (X 10^3)
	Dog 1			*A. Intramuscular Iron-Dextran*					Dog 2		
−20	4.40	7.4	28	0.6	24.2	−20	8.16	18.4	52.5	0.3	20.8
−3	5.29	7.1	23.5	2.2	20.8	−13	8.00	18.7	50.0	2.9	--
9	4.49	9.2	28.5	3.9	--	81	5.96	16.5	45.5	--	--
24	4.97	9.2	32	3.3	--	143	5.33	15.1	40.0	--	15.6
31	4.68	10.3	31	5.4	--	186	4.59	14.5	39.0	--	13.8
38	3.69	11.7	28.5	6.1	--	234	4.46	13.4	38.0	--	21.0
45	3.83	9.6	28	1.2	--	291	4.96	14.5	42.0	--	26.3
53	4.55	12.3	34	18.8	--						
81	4.20	12.5	35	--	--						
133	3.46	9.3	21.5	1.0	16.2						
186	3.72	11.2	32	--	10.8						
	Dog 3			*B. Intraperitoneal Iron-Dextran*					Dog 4		
−16	5.55	9.8	36.5	0.3	19.5	−12	5.24	12.3	33	1.0	26.0
−12	4.87	12.9	35.5	0.4	30.4	−3	4.29	10.2	28	11.9	15.2
−3	5.23	10.5	31.5	2.2	20.3	82	5.47	14.8	41	--	--
82	7.07	16.9	46.5	--	--	132	4.15	10.5	31	--	24.4
186	6.85	17.0	45.0	--	14.9						

Hematologic effects: No consistent effect of iron-dextran injections on red cell count, hemoglobin value, or packed cell volume was discerned in any of the dogs except Dog 1. This animal, which had an iron deficiency anemia at the beginning of the study, responded to intramuscular iron-dextran by a slow rise in hemoglobin and a delayed reticulocytosis. No appreciable change in white cell count was observed in any of the dogs. (Table I.)

Tests of hepatic function: Bromsulphalein retention was within normal limits in all dogs both before and approximately 2½ and 4½ months after injections of iron-dextran were begun. The serum bilirubin was also normal. Protein fractionation of serum from Dogs 1 and 2, as tabulated below (Table II), showed a definite fall in total protein, due largely to a fall in the albumin fraction. There was a corresponding elevation of serum globulins in the last two samples from Dog 2. Paper electrophoretic analysis confirmed these serum protein changes and demonstrated a diffuse increase of gamma globulins.

TABLE II. TOTAL AND FRACTIONAL SERUM PROTEIN ANALYSES OF DOGS GIVEN IRON-DEXTRAN INTRAMUSCULARLY

ANIMAL AND TIME OF STUDY	TOTAL PROTEIN (GM./100 ML.)	ALBUMIN (GM./100 ML.)	GLOBULIN (GM./100 ML.)
Dog 1			
Base line	5.9	2.9	3.0
2½ mo.	5.9	3.0	2.9
4½ mo.	4.5	2.0	2.5
Dog 2			
Base line	6.9	3.9	3.0
2½ mo.	5.9	2.8	3.1
4½ mo.	4.7	1.9	2.8
7½ mo.	5.8	1.5	4.3
9½ mo.	5.0	1.5	3.5

Miscellaneous observations: Fasting blood sugar values were within normal limits in all dogs. A nonprotein nitrogen determination, performed on plasma from Dog 2 just before death, when anuria and tarry diarrhea were present, was 26 mg. per 100 ml. Serum iron levels are tabulated (Table III). Although they were elevated in every dog, complete saturation of transferrin was not uniformly achieved—a finding which suggests that some of the iron was circulating as iron-dextran.

Tissue distribution of injected iron (Table IV): The greatest concentration of iron occurred in the liver, spleen, and peripancreatic lymph nodes where values were 50 to 75 times normal. Other organs had iron concentrations higher than normal, but the increase was most striking in tissues with a large reticuloendothelial component.

Postmortem findings: Autopsies were performed within 3 hours of death or after a period of 2 to 6 weeks' storage in a Deepfreeze. Since the postmortem findings in all dogs given iron-dextran were similar, a single description will be given, with salient exceptions noted.

The dogs showed marked emaciation and dull, lusterless hair. A scaling, lichenified skin rash was present in Dogs 3 and 4. Dark-brown pigmentation of the skin in an area

around the sites of injection was present in all animals. There was no free fluid in pleural, pericardial, or peritoneal cavities. The peritoneum and omentum of the dogs that received intraperitoneal injections were stained brown; no adhesions or peritonitis was present. A distinct, rust-brown color was noted in the spleen, lymph nodes, pancreas, and kidneys. The liver was a dark mahogany brown, filled the upper third of the abdomen, and accounted for 6.4 to 13.4 per cent of the animal's weight. Diffuse, black (iron sulfide) pigmentation was present in the intestines of dogs that had undergone postmortem autolysis. The duodenum of Dog 1 showed ascaris infestation. The bladder of Dog 2 was enormously distended and contained several sharp, granular calculi in the bladder neck. The thigh muscles of both dogs that received intramuscular injections were swollen, stained chocolate brown, and the fascial planes were indistinct. No abnormalities of the heart, lungs, great vessels, brain, or other organs were visible.

TABLE III. VALUES OF SERUM IRON, UNSATURATED IRON BINDING CAPACITY, AND TOTAL IRON BINDING CAPACITY IN DOGS GIVEN IRON-DEXTRAN PARENTERALLY

ANIMAL AND TIME OF STUDY	SERUM IRON (μg/100 ML.)	UNSATURATED IRON BINDING CAPACITY (μg/100 ML.)	TOTAL IRON BINDING CAPACITY* (μg/100 ML.)
Dog 1			
Base line	36	341	377
1 mo.	226	64	290
2½ mo.	434	90	524
4½ mo.	693	0	693
Dog 2			
Base line	214	233	447
2½ mo.	1,030	0	1,030
4½ mo.	420	0	420
7½ mo.	334	45	379
9½ mo.	314	4	314
Dog 3			
Base line	119	206	325
2½ mo.	486	9	495
Dog 4			
Base line	173	90	263
2½ mo.	1,720	0	1,720

*Total iron binding capacity is defined as the sum of serum iron and the unsaturated iron binding capacity.

TABLE IV. IRON CONCENTRATION (MG./GM. WET WEIGHT) OF TISSUES OBTAINED AT AUTOPSY OF NORMAL DOGS AND DOGS WITH IRON OVERLOAD

TISSUES	DOG 1	DOG 2	DOG 3	DOG 4	NORMAL DOGS (AVERAGE)
Liver	9.33	12.20	15.08	16.5	0.20
Spleen	5.79	8.23	8.32	4.87	0.24
Pancreas	----	0.13	0.47	0.61	0.06
Kidney	0.39	0.46	0.53	0.41	0.08
Heart	0.13	0.08	0.19	0.38	0.07
Lungs	0.27	0.41	0.18	0.48	0.25
Stomach	0.14	0.05	0.20	----	0.04
Peripancreatic node	12.7	23.8	19.8	18.40	0.06
Bone marrow	2.15	1.31	4.74	----	0.22
Muscle (skeletal)	0.10	0.05	0.163	0.10	0.04
Muscle (skeletal)*	17.15*	15.2*			
Skin (abdomen)	0.38	0.05	0.59	----	0.02
Skin (abdomen)*	----	0.43*	7.56*	1.27*	
Thyroid	----	0.08	----	----	0.06
Adrenal	----	0.42	1.57	----	0.07
Testis	0.18	----	0.50	----	0.01
Eye	0.02	0.01	0.06	----	0.01

*Sample taken from site of iron-dextran injections.

Microscopic examination of the liver showed large clumps of iron-filled macrophages (siderophages) aggregated within the sinusoids but relatively few in the fibrous tissue of the portal areas. Only occasional small granules of iron were seen in either the hepatic parenchymal cells or the bile duct epithelium. Slight atrophy of liver cell cords in the regions of maximal iron deposition was noted; there was no fibrosis or cirrhosis (Fig. 3).

The spleen of each dog contained large numbers of iron granules distributed almost exclusively within macrophages of the sinusoids in the red pulp, but few or none were found in the follicles and the capsule. Large deposits of granular iron in the fibroadipose tissue in the hilum of the spleen were seen in 2 animals (Fig. 4).

Peripancreatic lymph nodes of all dogs and the inguinal and iliac nodes of dogs receiving iron-dextran intramuscularly showed heavy infiltration of iron in the pulp but little in the follicles. Tremendous deposits of iron occurred in the sinusoidal areas and the perinodal fibroadipose tissue. Paratracheal and axillary nodes were pigmented but to a smaller extent. No fibrosis of any lymph node was seen.

No abnormalities were noted in the pancreas. Acinar and islet tissue appeared normal, and there was no fibrosis. Only an occasional siderophage was present in the interstitial tissues.

The kidneys showed moderately heavy accumulations of siderophages in the fibroadipose tissue about the pelvis but relatively little iron within the kidney substance. There was a moderate amount of finely granular iron in the glomerular tufts and in the epithelium of segments of the convoluted tubules (Fig. 5). The exact location of this segmental tubular involvement was not clear, but it appeared to be principally in the proximal segments. No histologic abnormalities other than the iron pigment were noted. The urinary bladder of Dog 3 showed numerous iron granules within macrophages in the adipose tissue beneath the serosa. Smaller amounts of iron were scattered in the interstitial tissue throughout the muscularis, particularly around blood vessels. In Dog 2, which had obstruction of the bladder neck due to calculi, there were focal areas of erosion in the bladder epithelium with underlying inflammation and hemorrhage into the lamina propria.

No abnormalities and no iron deposition were observed in the heart of any dog except Dog 3. This animal showed a moderate number of scattered siderophages in the connective tissue beneath the epicardium and endocardium and between the muscle fibers. Iron in the lungs was confined primarily to macrophages in alveolar septa which appeared to have been trapped in alveolar capillaries. A few siderophages were present in the fibrous tissue around the bronchi; others lay free in the alveolar lumens. Bronchitis and edema were present in Dog 4, and Dog 1 had an extensive bronchopneumonia.

Sections of bone marrow taken from ribs, sternum, vertebral body, and femur all showed moderate to heavy concentrations of granular iron in clumps, but no detectable cellular abnormality and no fibrosis. The structure of bone and cartilage was normal.

No iron was seen within the brain substance or in the choroid plexus. Rare macrophages in the leptomeninges contained small granules of iron. The eyes of Dog 2 showed no abnormalities.

Adrenals, thyroid, pituitary, and salivary glands showed no structural abnormalities. Small granules of iron were present in isolated macrophages of the interstitial connective tissue, but none was seen within epithelial cells.

Sections of muscle at sites of iron-dextran injection showed extensive areas of dense infiltration with macrophages containing large iron granules. These masses of iron displaced the muscle fibers but seemed to have incited no fibrous or foreign body reaction. The muscle fibers themselves appeared to be normal except for atrophy in some areas. Muscles in areas distant from sites of iron injection were normal and showed no iron deposition. Sections of diaphragm from dogs receiving iron-dextran intraperitoneally showed small to moderate numbers of coarse iron granules in the interstitial tissue between muscle bundles.

Siderophages in moderate numbers were scattered throughout the upper corium and were particularly concentrated around blood vessels and skin appendages. Considerable

Fig. 3.

Fig. 4.

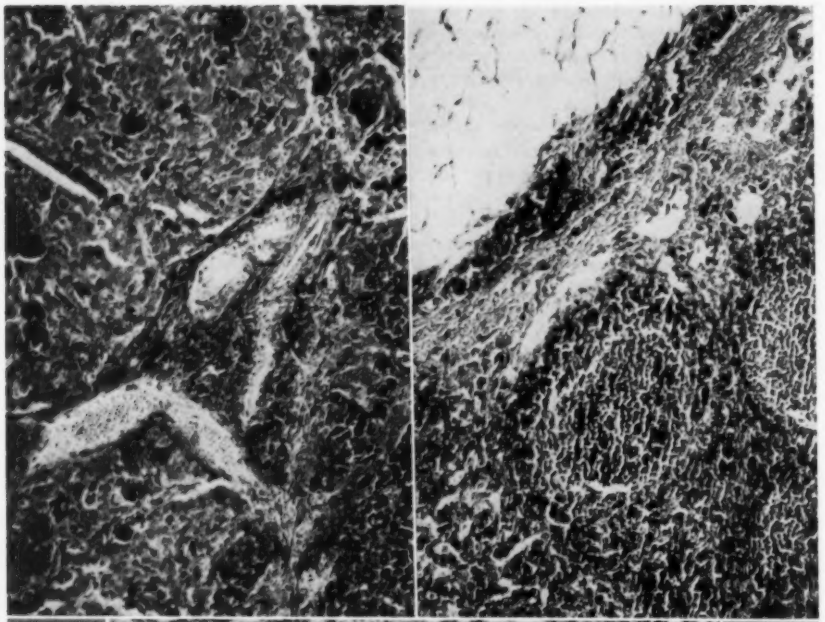

Fig. 5.

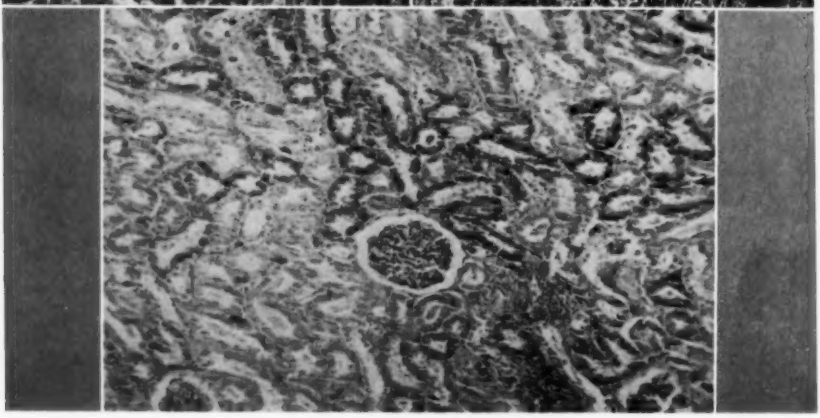

Fig. 3.—Liver from Dog 2 which received iron-dextran intramuscularly. The iron, which shows as black masses in the illustration, is concentrated in clumps of macrophages in the sinusoids and portal space. There is no fibrosis, injury to hepatic cells, or iron in bile duct epithelium, and only a small amount of iron in hepatic cells. Hematoxylin and eosin stain. (Magnification ×140; reduced ⅙.)

Fig. 4.—Spleen from Dog 2. The dark masses of iron are present in macrophages principally in the red pulp about the follicles but also in areolar tissue adherent to the capsule. Hematoxylin and eosin stain. (Magnification ×140; reduced ⅙.)

Fig. 5.—Kidney from Dog 2. Granules of iron are present in renal tubular cells and in endothelial cells of the glomerular tufts without inducing appreciable alterations in these tissues. Gomori's iron stain. (Magnification ×140; reduced ⅙.)

keratinization of the epithelium and plugging of the hair follicles with coarse clumps of keratin were seen.

Scattered siderophages were seen in the interstitial tissue of the testes in all dogs. The testes of Dog 1 showed some arrest of spermatogenesis, as evidenced by absence of spermatids, but there was no atrophy or fibrosis. Dog 2 showed active spermatogenesis. Testicular fibrosis and atrophy were present in the half-grown dog (No. 3). This animal also had moderate concentrations of siderophages throughout the fibrous tissue of the tunica vaginalis and epididymis. Except for scattered siderophages throughout the stroma of the prostate, this organ was normal in all animals.

There were slight to moderate numbers of siderophages in the connective tissue beneath the epithelium of the tongue in Dogs 2 and 3. Siderophages in the fibrous tissue of the lamina propria and adventitia of the esophagus were present only in the dogs that had received iron-dextran intraperitoneally. In the stomach and intestines the connective tissue of the lamina propria, muscularis, and subserosa showed moderate numbers of macrophages containing iron. Dogs 3 and 4, which had received intraperitoneal injections, showed larger concentrations of iron in the serosa than the animals that had received intramuscular injections. No epithelial iron deposition or lipochrome pigmentation was seen.

Huge deposits of hemosiderin accompanied by granular eosinophilic debris that may have represented the residue of edema or foreign body reaction were present in remnants of fibroadipose tissue of the omentum in the dogs injected intraperitoneally with iron-dextran. The gall bladders of these dogs showed large clumps of siderophages in the subserosal connective tissue and lymphatic spaces.

Certain findings warrant emphasis. There was no significant difference in iron distribution correlated with the route of iron-dextran administration. Iron deposition was confined almost exclusively to cells of the reticuloendothelial system. Parenchymal iron deposits were small and confined to epithelial cells of the liver, bile ducts, and renal tubules. Even when present in tremendous concentration, the iron produced no fibrous tissue or foreign body reaction; there was no cirrhosis of the liver or pancreatic fibrosis. Testicular atrophy was present in one dog and decreased spermatogenesis was observed in another; however, the amount of iron in the testes of these dogs was small, and the testicular changes were unlike those described by Nissim[2] in rats injected with iron-dextran. The histologic appearance of the tissues failed to show the cause of death. The bronchitis and bronchopneumonia in 2 dogs were probably terminal phenomena.

B. Saccharated Iron Oxide Iron Overload.—Dog 5, injected intravenously with saccharated iron oxide, had been a member of the colony for 2 years and had been used in several earlier experiments. One of these was an assay of the effects of 4-aminofolic acid with and without concomitant folic acid; no ill effects were seen. Prolonged phenylhydrazine-induced hemolytic anemia was subsequently produced in this dog, but he was allowed to recover completely before beginning this study. One month before iron injections were begun a laparotomy was performed, and biopsies for chemical iron analysis and histologic appraisal were taken of liver, spleen, pancreas, kidney, mesenteric lymph node, skeletal muscle, and skin. The histologic appearance of these tissues was normal except for the following: (1) iron was present in increased amounts principally in Kupffer cells of the sinusoids of the liver and in a few macrophages of the portal areas; (2) numerous siderophages were present in the red pulp of the

TABLE V. IRON ANALYSIS OF TISSUES OBTAINED AT BIOPSY AND AUTOPSY OF DOG 5 (GIVEN SACCHARATED IRON OXIDE INTRAVENOUSLY)

TISSUE	NORMAL Fe CONC. (MG./GM.)	PRE-INJECTION BIOPSY Fe CONC. (MG./GM.)	AUTOPSY Fe CONC. (MG./GM.)	ORGAN WEIGHT (GM.)	ESTIMATED ORGAN Fe CONTENT (GM.)	COMMENT
Liver	0.20	1.01	24.7	1,040	25.7	Body weight at autopsy—10 Kg.
Spleen	0.24	0.65	22.0	85	1.87	Fe given by injection—29 Gm.
Pancreas	0.06	0.03	0.63	25	0.02	Fe recovered. (Estimated*)—32 Gm.
Kidneys	0.08	0.18	0.82	75	0.06	
Heart	0.07	--	0.24	65	0.02	
Lymph node	0.06	0.08	35.0	10	0.35	
Muscle	0.04	0.06	0.11	3,500*	0.38	
Skin	0.02	0.06	0.40	700*	0.28	
Intestine	0.04	--	0.41	300	0.12	

*Assuming that: (1) muscle mass is 35 per cent of body weight; (2) skin mass is 7 per cent of body weight; and (3) approximately 3.2 Gm. of iron is present in bone marrow, lungs, and other tissues not sampled.

spleen; (3) fine granules of hemosiderin were seen in the epithelium of proximal convoluted tubules of the kidneys. The iron concentration of these tissues (Table V) is uniformly increased above normal values. The animal recovered uneventfully from the laparotomy and appeared to be in excellent condition at the start of iron injections.

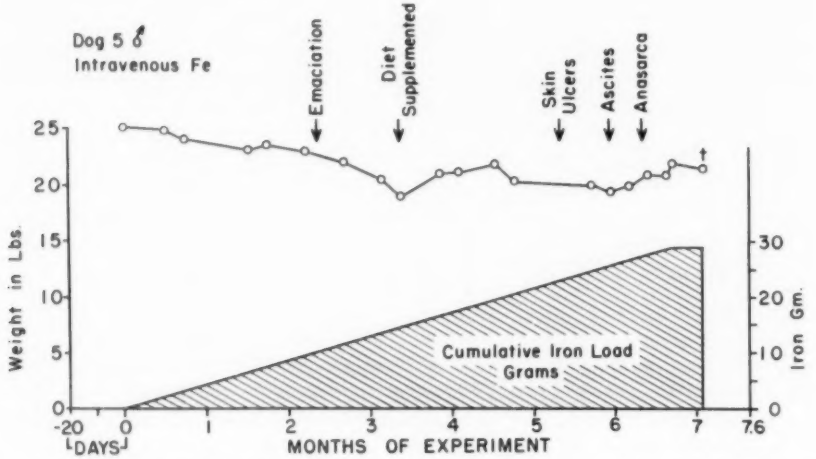

Fig. 6.—Correlation of body weight and toxic effects with cumulative iron load in dog that received saccharated iron oxide intravenously.

General effects: The dog remained in excellent condition throughout the first month of injections. In Fig. 6 are shown the weight loss and other physical signs which appeared with the accumulation of injected iron. Anorexia,

weight loss, emaciation, and unkempt, lusterless hair were prominent at 3 months. Supplementation of his regular diet with horse meat resulted in a temporary weight gain and improved appearance. At 5 months, however, ulcers appeared in the skin of the face and forelegs and failed to heal despite the use of penicillin injections, Aureomycin by mouth, and local antibiotic salves. Two weeks later ascites appeared, then edema of the feet and legs. Attempts to alleviate the ascites and edema by means of repeated injections of mercurial diuretics and 3 transfusions of plasma from a normal dog were unsuccessful. The animal died 13 days after his last iron injection, 7 months after the start of the experiment.

Laboratory observations: Since the effects of saccharated iron oxide on blood counts, serum iron, hepatic function, and blood sugar levels in an earlier series of dogs have been reported,[1] only a minimum of observations was made on this dog (Table VI). Progressive anemia began less than 2 months after the start of injections and continued until death. During the last 2 weeks of life the serum albumin, the total protein, and the free and esterified cholesterol were low, suggesting functional impairment of the liver. The serum iron did not rise to levels as high as those of dogs in another series which received Ferrivenin.[1] No evidence of diabetes was observed.

TABLE VI. LABORATORY DATA OF DOG 5 GIVEN SACCHARATED IRON OXIDE INTRAVENOUSLY

DAYS OF I.V. IRON INJECTIONS	RED CELL COUNT (MILLIONS)	HEMOGLOBIN (GM./100 ML.)	CELL VOLUME PER CENT	SERUM IRON (μg/100 ML.)
0	--	16.6	47	---
59	5.53	13.2	36	---
86	4.08	11.8	34	810
100	3.65	10.5	30	---
124	--	12.4	35	690
136	4.07	12.3	38	---
177	3.86	10.7	32	543
198	3.40	8.6	26	550
205	2.70	8.0	24	690
208	2.70	7.4	21	789

Miscellaneous tests were as follows. Day 198: total serum proteins, 4.9 Gm./100 ml.; albumin, 1.8 Gm./100 ml.; serum cholesterol (total), 47 mg./100 ml.; free, 22 mg./100 ml. Day 208: total serum proteins, 4.9 Gm./100 ml.; albumin, 2.0 Gm./100 ml.

Distribution of injected iron: The total iron found in the tissues of this dog was estimated at 28.8 of the 29 Gm. injected (Table V). An additional 3.2 Gm. of iron probably was present in the bone marrow, lungs, and other tissues not assayed, bringing the total recovery to 32 Gm. or 3 Gm. more than was injected. The most plausible explanation for this discrepancy is that this animal had increased body iron stores before the beginning of this study (as a result of receiving a prolonged course of phenylhydrazine to induce anemia in a previous study). The distribution of iron was similar to that of the dogs receiving iron-dextran and showed huge accumulations in liver, spleen, and peripancreatic lymph nodes and very little iron in tissues having a small reticuloendothelial component.

Postmortem findings: Gross examination revealed generalized muscle wasting, distention of the abdomen, and edematous extremities. Infected ulcers were present on the face

and hind legs; the hair was dry and lusterless; the skin and mucous membranes were rust-colored. Pink ascitic fluid (1,450 ml.) was present, but there was no free fluid in the pleural or pericardial cavities. The liver was large, firm, and chocolate brown. The spleen and peripancreatic lymph nodes were dark brown; the pancreas and kidneys were a lighter tan color. Except for a rusty pigmentation of the mucosa of the esophagus and stomach and the presence of a tapeworm in the upper ileum, the gastrointestinal tract was normal. Examination of the heart showed brown plaques on the endocardial surfaces of the mitral, aortic, and pulmonic valves and similar plaques in the aorta extending to a point 1 cm. below the origin of the subclavian artery. No abnormalities were seen on gross examination of the other organs.

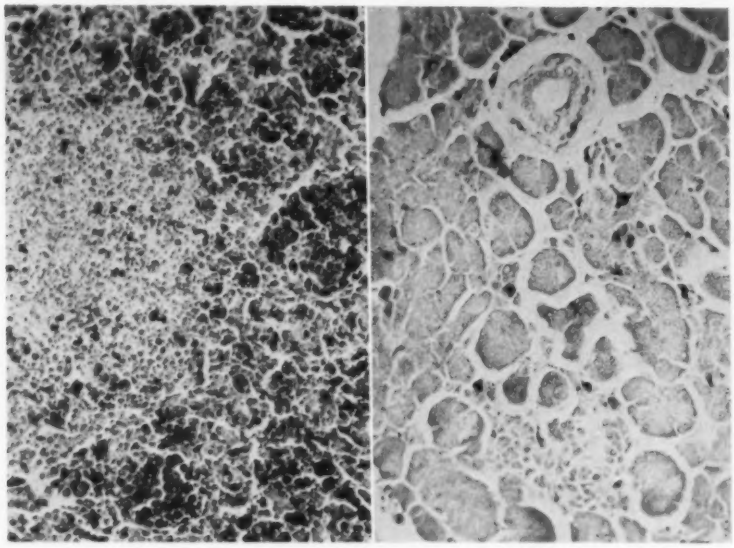

Fig. 7. Fig. 8.

Fig. 7.—Liver from Dog 5 which received saccharated iron oxide intravenously. Dark masses of iron are distributed in a manner similar to those in Fig. 3. There is a large area of bland necrosis with iron granules in its debris. Hematoxylin and eosin stain. (Magnification ×140; reduced ¼.)

Fig. 8.—Pancreas from Dog 5. There is no fibrosis and the epithelium of neither the islets nor the acini shows any changes. Very little iron is present, mostly as dark masses in the interstitial tissue. Gomori's iron stain. (Magnification ×230; reduced ¼.)

Microscopic examination showed the same general pattern of iron distribution as has been described previously.[1] The amount of iron deposition was greater, reflecting the three- to sixfold increase in dosage, but the predominant reticuloendothelial distribution and absence of fibrous tissue reaction to the iron were identical. Because of the similarity to the other dogs, only a description emphasizing differences will be given.

Iron deposits in the liver were confined to huge clumps of macrophages within the sinusoids and to smaller groups of distinct granules in the cytoplasm of the parenchymal cells; the bile duct epithelium was practically free of iron. Of particular interest were areas of focal necrosis 50 to 250 μ in diameter scattered throughout the liver (Fig. 7). In these areas there were destruction of liver cells, a slight increase in wandering macrophages, and a few polymorphonuclear leukocytes. The reticulum appeared to be preserved, and iron was present in macrophages or as free granules where cells had been destroyed. The remainder of the liver showed no increase in fibrous tissue or other abnormalities.

The spleen was massively infiltrated with siderophages predominantly within the reticulum of the red pulp, with little iron in the follicles. As was true of most organs examined, occasional iron-containing macrophages were present beneath the endothelium of blood vessels. The architecture of the pancreas was normal and showed no fibrosis. Iron deposition in this organ spared epithelial cells and was confined to interstitial tissue (Fig. 8).

The kidney glomeruli showed a peculiar and irregular thickening of the capillary basement membranes within the tufts, and contained relatively large accumulations of iron granules. This thickening seemed to have decreased the general vascularity of the glomerular tufts, although many capillaries were wide open and filled with erythrocytes. Small amounts of finely granular hemosiderin were present in the tubular epithelium, particularly of the proximal tubules (Fig. 9).

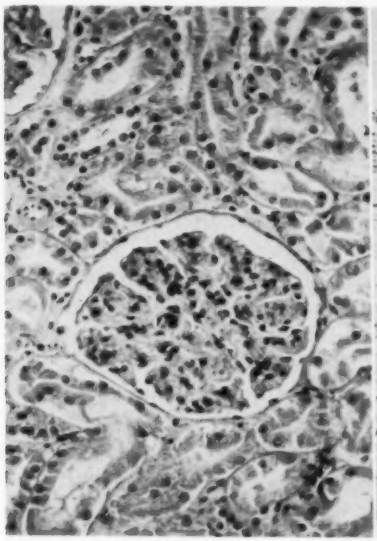

Fig. 9.

Fig. 10.

Fig. 9.—Kidney from Dog 5. The capillary basement membranes of the glomeruli are irregularly thickened and cause constriction of the lumens of the capillaries. Dark masses of iron are also present in the glomeruli but only fine granules are present in the epithelium of a few tubules. Hematoxylin and eosin stain. (Magnification ×270; reduced ¼.)

Fig. 10.—Aorta of Dog 5. An intimal plaque from just above the aortic valve shows the accumulation of macrophages filled with iron granules throughout its substance. Hematoxylin and eosin stain. (Magnification ×140; reduced ¼.)

In the heart siderophages were present almost exclusively in interstitial tissues of the myocardium and epicardium. In addition, there was deposition of siderophages, 4 or 5 cells thick, in the loose fibrous tissue immediately beneath the endocardium and the intima of the aorta which accounted for the plaques seen on gross examination (Fig. 10). Iron in the lungs was present only in siderophages trapped in alveolar capillaries.

Iron in the gastrointestinal tract was present mainly in macrophages in the lamina propria, with small collections of siderophages in the submucosa of the stomach and the subserosa of the small intestines. There was a brownish-yellow, refractile pigment that did not stain for iron but did stain positively with basic fuchsin in the interstitial tissue of the muscularis. This lipochrome or "hemofuscin pigment" was present in greatest quantities in the lower ileum but was also seen in the stomach, jejunum, colon, and interstitial tissue of the pancreas.

The testes showed scattered iron-containing macrophages in the interstitial tissue between the tubules but no atrophy of the tubules. Rare siderophages were present in the interstitial tissues of pituitary, adrenal and salivary glands; no pigmentation of the epithelium was seen.

Sections of vertebral and femoral bone marrow showed erythroid hypoplasia with slight fibrosis and a heavy infiltration of siderophages. Lymph nodes were filled with huge aggregates of siderophages, some of which spilled into surrounding alveolar tissue and a few into the interstitial areas of the compressed follicles.

Relatively small amounts of iron were found in muscle and skin, in siderophages about the blood vessels and hair follicles. A section of a chronic ulcer of the foreleg showed no deposition of iron in association with the inflammatory infiltrate.

No iron was seen in brain substance. A small number of siderophages was present in cells of the choroid plexus and the leptomeninges. The eyes contained moderate amounts of hemosiderin in the ciliary epithelium, in the stroma of the ciliary body, and in the choroid. The pigment epithelium showed swelling and degeneration, but the retina was free of abnormalities.

As in the dogs that received iron-dextran, this dog showed iron deposits primarily in the cells of the reticuloendothelial system, with parenchymal deposition only in the liver and kidney tubules. No cirrhosis or fibrosis was produced by the iron. Four findings in Dog 5 did not appear in the dogs which received iron-dextran. First, there were small scattered areas of focal necrosis of the liver of unknown significance. Second, lesions of the glomeruli similar to those described in other species of experimental animals as being due to small emboli caused by intravascular precipitation of saccharated iron oxide were observed.[3, 4] (The extensive renal lesions described by Ellis[5] in rats injected with saccharated iron oxide were not seen.) Third, hemofusein-like pigment was found in the pancreas and the muscularis of the gastrointestinal tract. The same pigment has been described in other dogs injected with saccharated iron oxide.[1] Golberg and associates[6] have suggested that a similar pigment produced in iron-dextran injected rats is related to a localized vitamin E deficiency. Finally, the iron deposits in various segments of the eye seemed to mark the early stages of degenerative ocular changes which have been described in other dogs injected with large amounts of iron.[7]

DISCUSSION

The cumulative dose of iron needed to produce recognizable toxic effects in these dogs was approximately 2 Gm. per kilogram of body weight; the lethal dose was 2.5 to 3.3 Gm. per kilogram. The dose was independent of the route of parenteral administration or type of iron compound used. The only report of iron overload of similar magnitude in dogs was that of Finch and Finch[8] who gave saccharated iron oxide and observed hepatic toxicity at doses of 2 Gm. per kilogram in dogs that survived for 2 years.

The way that the excess tissue iron produced the observed harmful effects is obscure. The anorexia, apathy, weight loss, and fall in serum albumin concentration do not implicate any specific mode of iron toxicity. They could be ascribed to malnutrition, but this label gives no insight into the role played by

the iron deposits. The course of the dogs that received iron-dextran suggests a saturation phenomenon. Early in the course of iron injections no ill effects were observed. After about 4 months, at levels of iron greater than 2 Gm. per kilogram, there seemed to be a breakdown of tolerance to the massive iron deposits with the appearance of toxic manifestations.

Finch and Finch have emphasized that iron introduced parenterally is largely captured by cells of the reticuloendothelial system so that the parenchymal cells are protected from damage and fibrous tissue response. If this explanation were wholly correct, one might expect that the terminal toxic effect could be correlated with a spillover of iron into parenchymal cells after the capacity of the reticuloendothelial system had been exceeded. No morphologic evidence for this phenomenon was found.

In addition to the other changes ascribed to malnutrition, the dog that received saccharated iron oxide developed ascites and anasarca, thereby suggesting liver failure as the cause of death. There is no doubt that the liver bears the brunt of iron deposition and may, therefore, sustain the greatest injury. Extremely low free and esterified serum cholesterol and low serum albumin concentrations in this animal were the only measured evidence of hepatic dysfunction; other functions such as bile pigment metabolism seemed to be reasonably intact. Examination of the liver at death gave little morphologic evidence of injury. Except for scattered, small areas of focal necrosis and the massive reticuloendothelial iron deposits, the liver did not appear seriously damaged. In the other dogs, even fewer abnormalities of the liver were noted.

Since there was no acceptable morphologic explanation in the liver or other tissues for the deterioration and death of these dogs, one may speculate about possible biochemical alterations that may have been involved. Little is known about iron toxicity at the molecular level. Ferritin, which is a usual accompaniment of hemosiderin in tissue iron storage, can under certain circumstances produce severe vasodepression and may lead to death.[9] Such a death is catastrophic, unlike the slow deterioration observed in these dogs. As another possibility, iron, a heavy metal, shares the property of binding sulfhydryl groups and may thus interfere with certain enzyme systems. Glycolysis of tissue homogenates is inhibited by ferrous sulfate,[10] and it has been postulated that pyruvate formation[11] and "enzymes in the Kreb's cycle"[12] are inhibited by iron in toxic amounts. The methods used in this experiment were not designed to test these hypotheses. It seems likely, however, that systemic toxic effects of iron overload invariably depend on interference with enzyme systems, whether or not morphologic changes are produced.

These data obtained from dogs may apply to human beings because of the many similarities of iron metabolism of the two species. The results emphasize the fact that excessive tissue iron deposits can lead to death by processes independent of the fibrosis, cirrhosis, and diabetes seen in human subjects with idiopathic hemochromatosis. Although no instance has yet been reported of harmful iron overload in human subjects as a result of administration of either saccharated iron oxide or iron-dextran, this possibility cannot be ignored. Even

the remote potentiality of fatal iron overload, as proved possible by these experiments, cautions against the indiscriminate use of these easily administered iron preparations.

SUMMARY AND CONCLUSIONS

The cumulative dose of parenterally administered iron required to produce death within 5 to 10 months in each of 5 dogs ranged from 2.5 to 3.3 Gm. per kilogram of body weight; toxic effects began to appear after 2 Gm. per kilogram had been given. The size of the lethal dose was not affected by the type of iron compound used (iron-dextran or saccharated iron oxide) or by the route of administration (intramuscular, intraperitoneal, or intravenous).

Massive tissue iron deposition occurred almost exclusively in cells of the reticuloendothelial system, with little parenchymal involvement. There was no fibrous tissue response to iron deposits and no evidence of hemochromatosis.

The mechanism by which the toxic effects were produced and the cause of death of these dogs have not been defined.

REFERENCES

1. Brown, E. B., Dubach, R., Smith, D. E., Reynafarje, C., and Moore, C. V.: Studies in Iron Transportation and Metabolism X. Long-term Iron Overload in Dogs, J. LAB. & CLIN. MED. **50**: 862-893, 1957.
2. Nissim, J. A.: Deposition of Iron in the Testes After Administration of an Iron-Dextran Complex, Lancet **1**: 701-703, 1955.
3. Polson, C. J.: The Fate of Colloidal Iron Administered Intravenously, J. Path. & Bact. **31**: 445-459, 1928.
4. Cappell, D. F.: The Late Results of Intravenous Injection of Colloidal Iron, J. Path. & Bact. **33**: 175-196, 1930.
5. Ellis, J. T.: Glomerular Lesions and the Nephrotic Syndrome in Rabbits Given Saccharated Iron Oxide Intravenously, J. Exper. Med. **103**: 127-144, 1956.
6. Golberg, L., Smith, J. P., and Martin, L. E.: Effects of Massive Iron Overload in the Rat, Nature **79**: 734, 1957.
7. Cibis, P. A., Brown, E. B., and Hong, S. M.: Ocular Effects of Systemic Siderosis, Am. J. Ophth. **44**: 158-172, 1957.
8. Finch, S. C., and Finch, C. A.: Idiopathic Hemochromatosis, an Iron Storage Disease, Medicine **34**: 381-430, 1955.
9. Mazur, A., Baez, S., and Schorr, E.: The Mechanism of Iron Release From Ferritin as Related to Its Biological Properties, J. Biol. Chem. **213**: 147-159, 1955.
10. Racker, E., and Krimsky, L.: Inhibition of Coupled Phosphorylation in Brain Homogenates by Ferrous Sulfate, J. Biol. Chem. **173**: 519-533, 1948.
11. Popper, H. P., and Schaffner, F.: Liver: Structure and Function, New York, 1957, Blakiston Company (Division of Doubleday & Company, Inc.).
12. Reissmann, K. R., and Coleman, T. J.: Acute Intestinal Iron Intoxication. II. Metabolic, Respiratory and Circulatory Effects of Absorbed Iron Salts, Blood **10**: 46-51, 1955.

SOME PHYSICOCHEMICAL PROPERTIES OF THE VITAMIN B_{12} BINDING SUBSTANCES OF NORMAL AND CHRONIC MYELOGENOUS LEUKEMIC SERA

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THE serum in chronic myelogenous leukemia (CML) has an increased concentration of vitamin B_{12}[1, 2] and an increased capacity to bind vitamin B_{12} as measured in vivo[3, 4] or in vitro.[1, 2, 5] By microbiologic assay of the electrophoretically separated fractions of normal and CML serum, vitamin B_{12} was found bound to the alpha-1 and alpha-2 globulins.[1, 6, 7] The same proteins were also involved in the in vitro binding of added vitamin B_{12}.[1, 6] When cobalt^{60}-labeled vitamin B_{12} ($B_{12}CO^{60}$) sufficient to saturate normal serum is added in vitro it is bound chiefly to the beta and alpha-2 globulins as measured by dialysis techniques. On the other hand, the high levels of added $B_{12}CO^{60}$ bound by CML serum is found on the alpha-1 and alpha-2 globulins.[8] In the present study we have investigated (a) the nature of the substances in normal and CML sera that bind $B_{12}CO^{60}$ and (b) some physicochemical characteristics of this binding reaction.

A preliminary report of this study has been published.[9]

METHODS

Serum.—Venous blood was collected from fasting control subjects and from CML patients. The blood was allowed to clot at room temperature and, 2 to 3 hours later, the serum separated and stored at -20° C. until used. Control sera were routinely pooled in groups of 3 to 4 and CML sera were occasionally pooled before use. Control subjects were laboratory personnel and patients in the hospital suffering from various types of functional disorders. CML sera were from patients who were in clinical and hematologic relapse.

Unsaturated Vitamin B_{12} Binding Capacity of Serum (UBBC).—The ability of normal or CML serum to bind added $B_{12}CO^{60}$ (UBBC) was determined by a slightly modified equilibrium dialysis technique described previously.[8] One milliliter of saline containing 29 mμg of $B_{12}CO^{60}$ was added to 4 ml. aliquots of normal and 1 or 2 ml. aliquots of CML sera. This amount of $B_{12}CO^{60}$ was in excess of the binding capacity of all sera except for one CML serum in which 58 mμg per milliliter of serum was required. The mixture was allowed to interact in a Visking dialysis bag for one hour at room temperature. Variations in the amount of serum used and in the total volume of the mixture did not affect the results provided an excess of $B_{12}CO^{60}$ was present. The serum $B_{12}CO^{60}$ mixture was then dialyzed for 48 hours at 4 to 6° C. against 300 ml. of 0.062 M phosphate buffer, pH 7.3. A protein-free blank containing buffer instead of serum was run concomitantly. The method of sample counting and the calculation of the UBBC from the experimental results was similar to that described previously.[8] All studies were performed in triplicate except for some experiments using CML sera

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Received for publication Sept. 11, 1958.

where duplicate samples only were analyzed. If duplicate or triplicate samples disagreed by more than 15 per cent the entire experiment was repeated. When serum whose binding capacity was saturated with added $B_{12}CO^{60}$ was required, the serum $B_{12}CO^{60}$ mixture was dialyzed against large volumes of 0.062 M phosphate buffer, pH 7.3 for 96 hours. In the estimation of the UBBC from such samples no correction for a protein-free blank was necessary.

Microbiologic Assay Measurements.—The *Euglena gracilis* assay was employed in these studies.[10] In this assay, one volume of diluted serum was added to an equal volume of basal growth medium. The pH of the basal medium as well as the serum-basal medium mixture is 3.6. The composition of this medium in grams per liter of water is as follows: H_3BO_3, 0.071; KH_2PO_4, 0.100; $MgSO_4 \cdot 7H_2O$, 0.100; L-glutamic acid, 10.000; DL-malic acid, 2.000; $CaCO_3$, 0.122; $ZnSO_4 \cdot 7H_2O$, 0.888; $MnSO_4 \cdot H_2O$, 0.031; $FeSO_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.004; $Na_2MoO_4 \cdot 2H_2O$, 0.005; $CoSO_4 \cdot 7H_2O$, 0.019; thiamine, 0.002. In this assay "free vitamin B_{12}" is defined as the vitamin available to *Euglena gracilis* without preliminary heating of the serum-basal medium mixture and "total vitamin B_{12}" as the vitamin available after heating of the serum-basal medium mixture at 100° C. for 17 minutes.[10] The "free" and "total" vitamin B_{12} activity was measured in: (A) serum to which excess $B_{12}CO^{60}$ had been added and had then been exhaustively dialyzed against 0.062 M phosphate buffer at pH 7.3. This serum contained the endogenous vitamin B_{12} plus the additional exogenous $B_{12}CO^{60}$ which was nondialyzable at pH 7.3. (B) Aliquots of the same serum to which $B_{12}CO^{60}$ had not been added but which had been dialyzed as serum A. This serum contained only the endogenous vitamin B_{12} activity. The amount of nondialyzable exogenous $B_{12}CO^{60}$ that was measured as "free" or "total" B_{12} in the *Euglena gracilis* assay could then be calculated from the following:

1. "Free" B_{12} activity of the exogenous $B_{12}CO^{60}$ = "Free" B_{12} activity (A) − "free" B_{12} activity (B)
2. "Total" B_{12} activity of the exogenous $B_{12}CO^{60}$ = "Total" B_{12} activity (A) − "total" B_{12} activity (B)

pH Experiments.—Sera were dialyzed for 72 hours against frequent changes of 0.15 M saline at 4° C. Aliquots of this sera were placed into dialysis bags and allowed to dialyze against buffers of varying pH values at constant ionic strength (0.15). In these experiments the $B_{12}CO^{60}$ was added to the dialysate rather than inside the bag. After dialysis for 72 hours the UBBC was determined. After dialysis, sera dialyzed against the pH 5.5 and 4.5 buffers became turbid but gross precipitation did not occur. The following buffers were used: (1) 0.15 M sodium borate, pH 9.2; (2) 0.062 M sodium phosphate, pH 7.3; (3) 0.15 M sodium acetate pH 5.5; (4) 0.15 M sodium acetate, pH 4.5; and (5) 0.15 M sodium formate, pH 3.6.

Serum containing bound $B_{12}CO^{60}$ was prepared by exhaustive dialysis as described above, and the UBBC was then determined. Aliquots of such sera were mixed with two volumes of the *Euglena gracilis* basal medium. The serum-basal medium mixture was then dialyzed against large volumes of the basal medium (pH 3.6). As a control two volumes of 0.062 M phosphate buffer pH 7.3 were added to aliquots of the same serum and the contents dialyzed against this buffer. The UBBC of the acidified and control serum were then redetermined after dialysis. The UBBC of the acidified serum listed in the Results was corrected for the slight decrease in the UBBC of the control serum.

Seromucoid Preparation.—The seromucoid, i.e., the fraction of serum soluble in 0.2 M sulfosalicylic acid[11] was prepared. One volume of 0.4 M sulfosalicylic acid was added to one volume of serum. One minute after precipitation of the serum proteins, the contents were centrifuged at 1,000 g 4° C., for 45 minutes. The supernatant containing the seromucoid was separated and then neutralized with 1 N NaOH. The seramucoid was precipitated using one-half volume of 5 per cent phosphotungstic acid in 2 N HCl.[11] $B_{12}CO^{60}$ binding by the neutralized supernatant both after sulfosalicylic acid precipitation and after phosphotungstic acid precipitation is shown in Table I. $B_{12}CO^{60}$ binding occurred in the sulfosalicylic acid

supernatant containing the seromucoid, but was virtually nil in the phosphotungstate supernatant in which the seromucoid was absent. Thereafter, $B_{12}CO^{60}$ binding by the neutralized sulfosalicylic acid supernatant was measured and taken to represent $B_{12}CO^{60}$ binding of the seromucoid.

TABLE I. THE BINDING OF $B_{12}CO^{60}$ BY THE SEROMUCOID FRACTION OF CML SERUM

	UBBC (mμg/ML.)
(A) CML serum	14.92
(B) Seromucoid fraction*	9.46
(C) Seromucoid supernatant†	0.01

*The supernatant after precipitation of the serum proteins from (A) with 0.2 M sulfosalicylic acid.

†The supernatant after precipitation of the seromucoid fraction from (B) with 5 per cent phosphotungstic acid in 2 N HCl.

The Effects of Heat.—Whole serum and the seromucoid fraction were heated for 3 minutes at 69 to 73° C. CML seromucoid was also heated for 10 minutes at 100° C. in a boiling water bath. All samples were then cooled to room temperature and $B_{12}CO^{60}$ binding measured.

RESULTS

Exchangeability of Bound $B_{12}CO^{60}$.—Carrier vitamin B_{12} was allowed to interact for one hour with serum containing bound $B_{12}CO^{60}$, and the UBBC was then redetermined by dialysis (Table II). No appreciable exchange of bound $B_{12}CO^{60}$ with carrier B_{12} was noted in either normal or CML sera. The rapidity of binding is indicated by the fact that carrier B_{12} failed to dislodge $B_{12}CO^{60}$ when the carrier vitamin was added to the serum one minute after the addition of the radiovitamin (Table III).

TABLE II. THE FAILURE OF CARRIER VITAMIN B_{12} TO EXCHANGE WITH $B_{12}CO^{60}$ PREVIOUSLY BOUND TO SERUM

SERUM	UBBC (mμg/ML.)	
	INITIAL BINDING	CARRIER B_{12}*
Normal	1.40	1.33
CML	12.46	12.79

*Four micrograms of carrier vitamin B_{12} was allowed to interact with sera containing previously bound $B_{12}CO^{60}$ for 1 hour at room temperature following which the UBBC was redetermined by dialysis.

TABLE III. THE RAPIDITY OF $B_{12}CO^{60}$ BINDING BY NORMAL AND CML SERUM

SERUM	UBBC (mμg/ML.)	
	CONTROL	CARRIER B_{12}*
Normal	1.53	1.43
CML	18.10	17.20

*One minute after the addition of 29 mμg $B_{12}CO^{60}$ to the serum, 500 mμg of carrier vitamin B_{12} was added. One hour later dialysis of all samples was begun.

Comparison of Euglena gracilis and Dialysis Binding Methods.—The radiovitamin bound to serum by dialysis at pH 7.3 was measured by the *Euglena gracilis* assay method. In normal sera 60 to 77 per cent of the radiovitamin nondialyzable at pH 7.3 was measured as "free" or unbound by *Euglena gracilis* assay whereas only 9 to 3 per cent was so measured in CML sera (Table IV).

However, expressed in absolute terms (millimicrograms per milliliter of serum), the amount of "free" $B_{12}CO^{60}$ was similar for both types of sera (Table IV). Since the serum is first acidified in the performance of the *Euglena gracilis* assay, the effect of an acid milieu on the $B_{12}CO^{60}$ nondialyzable at pH 7.3 was investigated.

TABLE IV. THE ABILITY OF EUGLENA GRACILIS TO UTILIZE NONDIALYZABLE $B_{12}CO^{60}$ OF NORMAL AND CML SERA

SERUM	"TOTAL" $B_{12}CO^{60}$ CONCENTRATION (mμg/ML.)	"FREE" $B_{12}CO^{60}$ CONCENTRATION (mμg/ML.)	$\frac{\text{"FREE" } B_{12}CO^{60}}{\text{"TOTAL" } B_{12}CO^{60}} \times 100$ (%)
Normal			
1	2.11	1.62	77
2	2.88	1.72	60
3	3.30	2.60	77
CML			
1	19.30	2.50	13
2	16.00	1.42	9
3	12.23	1.53	13

"Free" and "Total" $B_{12}CO^{60}$ in terms of *Euglena gracilis* assay as described in Methods.

TABLE V. THE EFFECT OF ACIDIFICATION ON THE NONDIALYZABLE $B_{12}CO^{60}$

SERUM	UBBC AT pH 7.3 (mμg/ML.)	UBBC AT pH 3.6* (mμg/ML.)	DECREASE IN UBBC AT pH 3.6	
			mμg/ML.	PER CENT
Normal				
1	2.19	1.14	1.05	48
2	1.52	0.80	0.72	47
CML				
1	8.65	7.70	0.95	11
2	5.93	5.19	0.74	12

*Serum $B_{12}CO^{60}$ complex acidified with the *Euglena gracilis* basal medium and then redialyzed to determine the UBBC as described in Methods.

TABLE VI. THE EFFECT OF ACIDIFICATION ON THE ABILITY TO BIND $B_{12}CO^{60}$

SERUM	UBBC (mμg/ML.)			
	pH 7.3	pH 5.5	pH 4.5	pH 3.6
Normal				
1	1.32	1.35	1.22	0.55
2	1.02	0.98	0.88	0.50
3	1.00	1.10	0.83	0.28
4	1.59	—	—	0.81
5	1.54	—	—	0.46
CML				
1	9.78	—	—	8.84
2	11.89	—	—	12.90
3	54.32	—	—	53.00

The above pH levels maintained by buffers of similar ionic strength (0.15) as described in Methods.

Effect of pH.—Sera containing $B_{12}CO^{60}$ nondialyzable at pH 7.3 was acidified and then dialyzed against the *Euglena gracilis* basal medium pH 3.6. A large fraction (47 to 48 per cent) of the $B_{12}CO^{60}$ nondialyzable at pH 7.3 became dialyzable or unbound on acidification (Table V). On acidification of CML sera a much smaller fraction of the $B_{12}CO^{60}$ was rendered dialyzable. The

effect of varying pH levels (3.6 to 9.2) on $B_{12}CO^{60}$ binding was investigated. Binding by normal sera was not affected by pH levels from 5.5 to 9.2. At pH 4.5 a slight decrease in $B_{12}CO^{60}$ binding was found while at pH 3.6 a marked decline occurred (Table VI). By contrast, $B_{12}CO^{60}$ binding by CML sera was unaffected at pH levels of 3.6 (Table VI).

TABLE VII. THE BINDING OF $B_{12}CO^{60}$ BY THE SEROMUCOID FRACTION OF NORMAL AND CML SERA

SERUM	SERUM UBBC (mμg/ML.)	SEROMUCOID UBBC (mμg/ML.)	$\frac{\text{SEROMUCOID UBBC}}{\text{SERUM UBBC}} \times 100$ (%)
Normals (6)*			
Mean ± S.D.	2.03 ± 0.14	0.26 ± 0.10	13 ± 5
Range	1.52 - 2.64	0.13 - 0.37	5 - 18
CML			
1	5.81	2.78	48
2	10.19	7.50	74
3	8.29	6.28	76
4 a)†	14.92	9.46	63
b)	28.00	24.50	88
5	4.72	1.46	31
6	4.84	2.17	45
Mean ± S.D.	10.97 ± 8.33	7.74 ± 7.97	65 ± 20
Range	4.72 - 28.00	1.46 - 24.50	31 - 88

*Refers to number of patients.
†Same patient studied during different relapses.

TABLE VIII. THE EFFECT OF THE PREHEATING OF SERA AND THEIR SEROMUCOID FRACTIONS ON THEIR ABILITY TO BIND $B_{12}CO^{60}$

SERUM	UBBC (mμg/ML.) CONTROL	UBBC (mμg/ML.) HEATED	$\frac{\text{UBBC AFTER HEAT}}{\text{CONTROL UBBC}} \times 100$ %
Normal Sera			
1	2.34	1.19	51
2	1.52	0.70	46
3	1.72	0.61	36
4	2.08	0.84	40
5	1.87	0.69	37
CML Sera			
1	5.81	4.89	84
2	8.10	6.59	81
3	10.19	8.60	84
4	23.90	21.50	90
CML Seromucoid			
1 a)	4.65	4.35	94
b)	4.65	3.80	82
2 a)	2.17	2.17	100
b)*	2.17	5.33	246
3 (1)	1.46	1.89	130
4 (1)	23.90	22.90	96

*Samples heated for 10 minutes at 100° C.; all other samples heated at 69 to 73° C. for 3 minutes.

Seromucoid Binding.—The binding of $B_{12}CO^{60}$ by the seromucoid fraction of normal and CML sera is shown in Table VII. A small amount of $B_{12}CO^{60}$ was bound by the seromucoid of normal sera (mean of 0.26 mμg per milliliter equal to 13 per cent of total serum binding). In CML sera this binding was increased about thirty fold (mean 7.74 mμg per milliliter equal to 61 per cent of

total serum binding). The binding of $B_{12}CO^{60}$ by the CML seromucoid was not affected by acid pH levels as low as 3.6. Precipitation of the normal or CML seromucoid-vitamin B_{12} complex with 5 per cent phosphotungstic acid resulted in 95 to 100 per cent recovery of the radiovitamin in the washed precipitate.

The Effect of Heat.—The effect of preheating the serum and/or seromucoid on the subsequent ability to bind added $B_{12}CO^{60}$ is shown in Table VIII. Heating of normal sera to 69 to 73° C. for 3 minutes resulted in a 49 to 64 per cent decrease in binding as compared to a 10 to 19 per cent decrease in binding in CML sera. Heating of CML seromucoid to 69 to 73° C. had no effect on $B_{12}CO^{60}$ binding, and heating to 100° C. had no effect or actually increased $B_{12}CO^{60}$ binding in 3 of the 4 sera tested.

DISCUSSION

A large portion (60 to 77 per cent) of the $B_{12}CO^{60}$ bound by normal serum at pH 7.3, i.e., the nondialyzable radiovitamin, was measured as "free" or unbound in the *Euglena gracilis* assay system. In this assay, performed at a pH of 3.6, "free" B_{12} is defined as the vitamin available to *Euglena gracilis* without preliminary heating of the serum-basal medium mixture. However, at such acid pH levels, 47 to 48 per cent of the $B_{12}CO^{60}$ nondialyzable at pH 7.3 became dialyzable or unbound. Thus, the ready availability for *Euglena gracilis* of a large fraction of the bound $B_{12}CO^{60}$ is due to the acid milieu of the assay system. Since the amount of the bound $B_{12}CO^{60}$ measured as "free" by *Euglena gracilis* exceeded the amount of bound $B_{12}CO^{60}$ rendered dialyzable by acidification, direct utilization of bound $B_{12}CO^{60}$ by *Euglena gracilis* without preliminary heating must also occur. Gräsbeck[12] has shown that vitamin B_{12} bound to gastric juice was unavailable for adsorption by *Lactobacillus leichmannii* at pH 7 to 8 but was readily adsorbed below pH 5. Furthermore, it has been shown that other microorganisms can utilize the vitamin B_{12} bound to intrinsic factor preparations without preliminary treatment at pH levels above 6.6.[13, 14] The data cited above emphasize that what is measured as "bound" vitamin in a dialysis or an ultrafiltration system may not be "bound" in a microbiologic assay system. The importance of pH in any type of assay system for the measurement of serum B_{12} binding is also indicated by the present findings. The variable results obtained by other investigators as to the principal binding proteins of normal serum for added B_{12} can probably be ascribed to the different methods of determining vitamin B_{12} binding employed in these studies.[6, 8]

Little, if any, of the vitamin B_{12} of normal serum is measured as "free" by *Euglena gracilis* assay.[6, 15] Thus, the endogenously bound vitamin differs from the vitamin bound in vitro with respect to its availability for *Euglena gracilis*. Differences in the chemical nature of the binding between the in vivo and in vitro situations are suggested by this finding. Furthermore, in vivo the vitamin is primarily associated with the alpha-1 and alpha-2 globulins,[6, 7] whereas in vitro the vitamin is primarily bound by the beta and alpha-2 globulins of normal serum.[8] Doubt as to the physiologic significance of the in vitro binding is suggested by the above findings. Thus, the binding of $B_{12}CO^{60}$ by the beta

globulins in the present in vitro system may only represent a "spill-over" from the saturated binding sites on the physiologic binding proteins, i.e., the alpha-1 and alpha-2 globulins. Attempts to check this hypothesis using concentrations of $B_{12}CO^{60}$ within the binding capacity of the alpha globulins have been unsatisfactory due to technical limitations. However, small amounts of "free" B_{12} as measured by *Euglena gracilis* can be found in normal sera[6, 15] and B_{12} activity has been found associated with the beta globulins and albumin fractions of normal serum.[6, 7] Thus, the binding of $B_{12}CO^{60}$ by the beta globulins and albumin fractions observed in vitro also occurs in vivo. The physiologic significance of these findings remains to be elucidated. By contrast to normal sera, the $B_{12}CO^{60}$ bound in vitro by CML sera is largely unavailable to *Euglena gracilis* without preliminary heating, and thus is similar in this respect to the vitamin bound in vivo.[1, 2]

The seromucoid fraction of CML serum was capable of binding large amounts of $B_{12}CO^{60}$ in CML patients. In some sera, the seromucoid accounted for the entire increase in $B_{12}CO^{60}$ binding over normal values. Two factors relating to the method of preparation of the seromucoid could influence the amount of $B_{12}CO^{60}$ bound by the seromucoid. Following perchloric acid precipitation of the serum proteins, approximately 30 per cent of the seromucoid is lost by coprecipitation.[11] Secondly, sulfosalicylic acid may liberate the endogenous vitamin B_{12} from the precipitated serum proteins with the liberated vitamin then bound by the seromucoid. Since both factors would decrease the binding capacity for additional $B_{12}CO^{60}$, the $B_{12}CO^{60}$ binding by the seromucoid fraction may be even higher than that measured.

The seromucoid fraction of serum moves with the alpha-1 and alpha-2 globulins on electrophoresis at pH 8.6.[16, 17] The alpha-1 and alpha-2 globulins are also associated with (1) the endogenously bound vitamin B_{12} of normal and CML serum[1, 6, 7] (2) the vitamin B_{12} bound following parenteral administration of the vitamin to normal subjects and patients with CML,[18] and (3) the exogenous $B_{12}CO^{60}$ bound in vitro by CML sera.[8] These findings suggest that the seromucoid, or a moiety thereof, may be responsible for the binding of the endogenous and exogenous vitamin B_{12} of CML serum. By the use of anion ion exchange resins, it has been established that the endogenous vitamin B_{12} of CML serum is not bound by the alpha-1 acid glycoprotein,[19] the main protein constituent of the seromucoid.[16] Since the seromucoid contains at least 3 different proteins,[16] the endogenous B_{12}-binding protein could still be a constituent of the seromucoid.

The increased $B_{12}CO^{60}$ binding capacity of the CML seromucoid can be due to: (1) an increase in the concentration of a B_{12}-binding substance present in the normal seromucoid; (2) an increase in the number of B_{12} binding sites of a B_{12}-binding protein normally found in the seromucoid; or (3) a B_{12}-binding substance foreign to the normal seromucoid. The increase in the concentration of the seromucoid fraction in CML[20, 21] is in favor of the first hypothesis. However, an increase in seromucoid concentration has been found in many diseases in which serum B_{12} binding is not increased.[20, 21] The present data is obviously insufficient to establish which of these hypotheses is correct.

The seromucoid fraction of serum contains a sturdy group of substances as demonstrated by the various methods used in its separation from the serum proteins, i.e., deproteinization of the serum by heating[22, 23] or precipitation of serum proteins with sulfosalicylic acid or perchloric acid.[11] Similarly, the B_{12} binding properties of these substances are not affected by relatively drastic chemical treatment, i.e., heating to 100° C. or acidification. Furthermore, the $B_{12}CO^{60}$-seromucoid complex is not disrupted when precipitated by 5 per cent phosphotungstic acid in 2 N HCl. The minimal effects of heat and of acidification on $B_{12}CO^{60}$ binding by CML whole sera are due to the increased binding by the resistant seromucoid fraction. The unavailability of the bound $B_{12}CO^{60}$ of CML sera for *Euglena gracilis* without heating is probably also explained by the increased seromucoid binding.

It is of interest that two substances with high vitamin B_{12} binding properties are mucoproteins, i.e., the intrinsic factor of Castle[24, 25] and the sow milk whey protein.[26] Evidence both for[27-30] and against[31-33] the presence of an extragastric intrinsic factor-like substance present in normal serum but absent in pernicious anemia serum has been presented. The available data do not suggest any relationship of the $B_{12}CO^{60}$-binding substances of the CML seromucoid to intrinsic factor. Thus, heating to 100° C. did not affect its $B_{12}CO^{60}$ binding ability in contrast to its effect in gastric juice.[34] An actual increase in binding by some seromucoid fractions was noted after heating, an effect not found with any purified or crude intrinsic factor preparation.[35] The substances of CML serum with increased $B_{12}CO^{60}$ binding properties move as alpha-1 and alpha-2 globulins on electrophoresis at pH 8.6,[8] whereas the intrinsic factor activity in human gastric juice was found slightly ahead of the beta globulins under similar conditions.[35]

SUMMARY AND CONCLUSIONS

1. Binding of added cobalt^{60}-labeled vitamin B_{12} ($B_{12}CO^{60}$) by normal and CML sera was studied by dialysis techniques. Added $B_{12}CO^{60}$ was rapidly bound and once bound did not exchange with additional "carrier" vitamin B_{12} in both normal and CML sera.

2. Of the bound $B_{12}CO^{60}$ of normal sera, i.e., the $B_{12}CO^{60}$ nondialyzable at pH 7.3, 60 to 77 per cent was measured as "free" by *Euglena gracilis* assay, whereas only 9 to 13 per cent was so measured in CML sera. At the acid pH at which the *Euglena gracilis* assay is performed (pH 3.6), 47 to 48 per cent of the bound $B_{12}CO^{60}$ of normal sera became dialyzable as compared to 11 to 12 per cent in CML sera.

3. $B_{12}CO^{60}$ binding by normal sera was unaffected by pH levels above 4.5 but a marked decline in binding was found at pH 3.6. In contrast, at pH 3.6, $B_{12}CO^{60}$ binding by CML sera was unaffected.

4. Preheating of normal sera to 69 to 73° C. for 3 minutes decreased $B_{12}CO^{60}$ binding by 49 to 64 per cent as compared to 10 to 19 per cent in CML sera.

5. $B_{12}CO^{60}$ binding by the seromucoid fraction of normal serum was equal to an average of 0.26 mμg per milliliter, constituting 13 per cent of the total serum binding, while binding by the CML seromucoid was equal to 7.74 mμg per milliliter, constituting 61 per cent of the total serum binding. Binding by the CML seromucoid was not affected by acidification or heating to 100° C.

6. Differences noted between normal and CMI sera with respect to (1) the effects of acidification and heating on $B_{12}CO^{60}$ binding and to the (2) availability of bound $B_{12}CO^{60}$ for *Euglena gracilis* are due to the increased binding by the CML seromucoid. The increased in vitro binding ability of CML serum for added $B_{12}CO^{60}$ is due to the increased $B_{12}CO^{60}$ binding by the seromucoid fraction of serum; the increased in vivo binding capacity of CML sera for vitamin B_{12} may be attributed to a similar mechanism.

We are indebted to Dr. N. S. Ritter, Merck & Company, Inc., who kindly supplied the cobalt^{60}-labeled vitamin B_{12}.

REFERENCES

1. Beard, M. F., Pitney, W. R., and Sanneman, E. H.: Serum Concentrations of Vitamin B_{12} in Patients Suffering From Leukemia, Blood **9**: 789, 1954.
2. Mollin, D. L., and Ross, G. I.: Serum Vitamin B_{12} Concentrations in Leukaemia and in Some Other Haematological Conditions, Brit. J. Haemat. **1**: 155, 1955.
3. Mollin, D. L., Pitney, W. R., Baker, S. J., and Bradley, J. E.: The Plasma Clearance and Urinary Excretion of Parenterally Administered $^{58}CoB_{12}$, Blood **11**: 31, 1956.
4. Miller, A., Corbus, H. F., and Sullivan, J. F.: The Plasma Disappearance, Excretion and Tissue Distribution of Cobalt^{60}-Labelled Vitamin B_{12} in Normal Subjects and Patients With Chronic Myelogenous Leukemia, J. Clin. Invest. **36**: 18, 1957.
5. Raccuglia, G., and Sacks, M. S.: Vitamin B_{12} Binding Capacity of Normal and Leukemic Sera, J. Lab. & Clin. Med. **50**: 69, 1957.
6. Pitney, W. R., Beard, M. F., and Van Loon, E. J.: Observations on the Bound Form of Vitamin B_{12} in Human Serum, J. Biol. Chem. **207**: 143, 1954.
7. Heinrich, H. C., and Erdmann-Oehlecker, S.: Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. II. Die Intravitale Bindung (Transport) der B_{12}-Vitamine an die Serumproteinfraktionen bie Hämoblastosen, Clin. chim. acta **1**: 311, 1956.
8. Miller, A.: The In Vitro Binding of Cobalt^{60}-Labelled Vitamin B_{12} by Normal and Leukemic Sera, J. Clin. Invest. **37**: 556, 1958.
9. Miller, A., and Sullivan, J. F.: Increased Binding of Serum Mucoproteins in Chronic Myelogenous Leukemia, J. Clin. Invest. **37**: 917, 1958.
10. Ross, G. I. M.: Vitamin B_{12} Assay in Body Fluids Using Euglena Gracilis, J. Clin. Path. **5**: 250, 1952.
11. Winzler, R. L., Devor, A. W., Mehl, J. W., and Smyth, I. M.: Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation, J. Clin. Invest. **27**: 609, 1948.
12. Grüsbeck, R.: The Vitamin B_{12}-Binding Principle of Human Gastric Juice: Influence of pH on the Bacterial Adsorption of Free and Bound B_{12}, Scandinav. J. Clin. & Lab. Invest. **9**: 50, 1957.
13. Bird, O. D., and Hoevet, B.: The Vitamin B_{12}-Binding Power of Proteins, J. Biol. Chem. **190**: 181, 1951.
14. Gregory, M. E., and Holdsworth, E. S.: Some Observations on the Measurement of "Binding" of Cyanocobalamin by Intrinsic Factor Preparations, Biochem. J. **66**: 456, 1957.
15. Mollin, D. L., and Ross, G. I. M.: The Vitamin B_{12} Concentrations of Serum and Urine of Normals and of Patients With Megaloblastic Anaemias and Other Diseases, J. Clin. Path. **5**: 129, 1952.
16. Mehl, J. W., Humphrey, J., and Winzler, R. J.: Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins From Perchloric Acid Filtrates of Plasma, Proc. Soc. Exper. Biol. & Med. **72**: 106, 1949.
17. Markham, R. L.: A Modified Method of Two-Dimensional Zone Electrophoresis Applied to Mucoproteins in Serum and Urine, Nature **177**: 125, 1956.
18. Heinrich, H. C., and Erdmann-Oehlecker, S.: Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. III. Resorption, Blutverteilung, Serumproteinbindung, Retention und Exkretion der B_{12}-Vitamine bei Hämoblastosen Nach Oraler und Parenteraler B_{12}-Applikation, Clin. chim. acta **1**: 326, 1956.

19. Mendelsohn, R. S., Watkin, D. M., Horbett, A. P., and Fahey, J. L.: Identification of the Vitamin-B_{12} Binding Protein in the Serum of Normals and Patients With Chronic Myelocytic Leukemia, Blood **13:** 740, 1958.
20. Greenspan, E. M.: Survey of Clinical Significance of Serum Mucoprotein Level, Arch. Int. Med. **93:** 863, 1954.
21. Moschides, E., Stefanini, M., Magalini, S. I., and Kistner, S. A.: Content and Composition of the Mucoprotein Fraction of Human Serum (Seromucoid) in Disease, With Special Reference to Hematologic Disorders, J. Clin. Invest. **37:** 127, 1958.
22. Bywaters, H. W.: Über Seromucoid, Biochem. Ztschr. **15:** 322, 1909.
23. Staub, A. M., and Rimington, C.: Preliminary Studies on the Carbohydrate-Rich Fractions of Ox Serum, Biochem. J. **42:** 5, 1948.
24. Glass, G. B., Boyd, L. J., Rubenstein, M. A., and Svigals, C. S.: Relationship of Glandular Mucoprotein From Human Gastric Juice to Castle's Intrinsic Antianemic Factor, Science **115:** 101, 1952.
25. Latner, A. L., and Ungley, C. C.: Intrinsic Factor Activity in Solid Material, Brit. Med. J. **1:** 1448, 1953.
26. Gregory, M. E., and Holdsworth, E. S.: Some Properties of the Cyanocobalamin-Protein Complex From Sow's Milk and the Mode of Linkage of Cyanocobalamin With Protein, Biochem. J. **59:** 335, 1955.
27. Callender, S. T., and Lajtha, L. G.: On the Nature of Castle's Hematopoietic Factor, Blood **6:** 1234, 1951.
28. Lajtha, L. G.: An Inhibitory Factor in Pernicious Anaemia Serum, Clin. Sc. **9:** 287, 1950.
29. Thompson, R. B.: Observations on the Effects of Vitamin B_{12}, Liver Extracts, Folic Acid and Thymine on the Maturation of Megaloblasts in Culture, Blood **7:** 522, 1952.
30. Pendl, I., and Franz, W.: Transformation of Megaloblasts to Normoblasts by Cultivating Human Bone Marrow in Presence of Vitamin B_{12} and Vitamin B_{12}-Binding Protein, Nature **181:** 488, 1958.
31. Horrigan, D. L., Jarrold, T., and Vilter, R. W.: Direct Action of Vitamin B_{12} Upon Human Bone Marrow. The Effect of Instillations of Vitamin B_{12} and Folic Acid Into Bone Marrow as Studied by Nucleic Acid Staining Techniques, J. Clin. Invest. **30:** 31, 1951.
32. Swan, H. T., Reisner, E. H., Jr., and Silverman, M.: The Effect of Various Metabolites on the Growth of Marrow Cells In Vitro, Blood **10:** 735, 1955.
33. Thomas, E. D., and Lochte, H. L., Jr.: Studies on the Biochemical Defect of Pernicious Anemia. I. In Vitro Observations on Oxygen Consumption, Heme Synthesis and Deoxyribonucleic Acid Synthesis by Pernicious Anemia Bone Marrow, J. Clin. Invest. **37:** 166, 1958.
34. Bunge, M. B., and Schilling, R. F.: Intrinsic Factor Studies. VI. Competition for Vit. B_{12} Binding Sites Offered by Analogues of the Vitamin, Proc. Soc. Exper. Biol. & Med. **96:** 587, 1957.
35. Gräsbeck, R.: Studies on the Vitamin-B_{12} Binding Principle and Other Biocolloids of Human Gastric Juice, Acta med. scandinav. Suppl. 314, 1956.

LABORATORY METHODS

A CAPILLARY TEST TUBE TECHNIQUE FOR THE DETERMINATION OF CAPILLARY WHOLE BLOOD PROTHROMBIN TIME

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THE repeated determination of the prothrombin time in infants, especially in the first few months of life, presents serious problems of technique. The repeated sampling of the usual amounts of blood required for the Link-Shapiro modification of the Quick test for prothrombin, as is generally used for this determination in our laboratory, requires repeated venepunctures, with the withdrawal of amounts of blood that are proportionally huge in relation to the small reserve available to the newborn infant. Since such tests are most often required of the abnormal infant, in contrast to a healthy baby able to withstand such vigorous testing, much investigation has gone into this phase of minimizing the trauma of the test to the infant. Several different types of micromethod determination of the prothrombin time, especially for use in infants, have been described in an effort to solve the dilemma presented.[1-9] However, these tests prove quite complex and cumbersome in their requirement for the exacting care and stringent conditions in handling and testing the small quantities of blood or plasma sample, and thus none offer a convenient and easily available bedside method for the accurate and immediate determination of the prothrombin time of the blood. This laboratory investigated the accuracy and utility of a bedside capillary test tube technique suggested by one of us (E. G.) for such a purpose.

METHODS

A clean glass capillary tube, measuring 1 mm. internal diameter and 3 cm. long, is marked off in 1 cm. intervals. A room temperature mixture of 0.0125 M calcium chloride and thromboplastin solution* is drawn up to the 2 cm. marking and then very freely flowing blood, as obtained from a deep skin puncture in a digital tuft, ear lobe, or heel, is drawn to the 3 cm. mark. The tube is then immediately grasped between the thumb and forefinger to close off the system and inverted repeatedly until the end point of a definite precipitate is reached. This precipitate is easily identified with practice. The exact

From the Departments of Medicine and Laboratories, Beth Israel Hospital, Newark, N. J.
Aided by a grant from the Newark Beth Israel Hospital Research Foundation.
Received for publication May 22, 1958.
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TABLE I. A COMPARISON OF PROTHROMBIN TIME DETERMINATIONS BETWEEN THE MODIFIED QUICK TEST USING PLASMA AND THE CAPILLARY TEST TUBE USING OXALATED VENOUS AND CAPILLARY BLOOD IN INFANTS

PATIENT NUMBER	PROTHROMBIN TIME IN SECONDS		
		CAPILLARY TEST TUBE METHOD	
	PLASMA METHOD	VENOUS OXALATED BLOOD	CAPILLARY BLOOD
1	22	18	16
2	20	18	17
3	17	17	17
4	26	22	23
5	15	16	15
6	15	20	19
7	15	18	17
8	16	18	18
9	16	17	17
10	17	19	18
11	16	19	17
12	16	16	18
13	22	20	19
14	19	20	19
15	18	21	19
Mean ± S.D. of Mean	18.6 ± 1.9	18.0 ± 3.6	18.0 ± 1.7

TABLE II. COMPARISON OF PROTHROMBIN TIME DETERMINATIONS BETWEEN THE MODIFIED QUICK TEST USING PLASMA AND THE CAPILLARY TEST TUBE USING THE SAME OXALATED VENOUS BLOOD SAMPLE

PER CENT DILUTION	PLASMA METHOD (REAGENTS AND PLASMA AT 37° C.)	CAPILLARY TEST TUBE METHOD: REAGENTS AND WHOLE BLOOD AT		
		22° C.	30° C.	37° C.
100	15*	15*	19*	15*
75	19	19	21	16
57	21	21	22	18
42	23	21	23	22
32	28	22	24	24
24	32	24	29	26
18	38	25	30	32
13	45	34	35	37
10	50	42	52	45

*Values given in seconds.

TABLE III. A COMPARISON OF CAPILLARY TUBE PROTHROMBIN TIME DETERMINATIONS BETWEEN OXALATED VENOUS BLOOD AND CAPILLARY BLOOD IN ADULTS

PATIENT NUMBER	PROTHROMBIN TIME IN SECONDS IN DUPLICATE	
	OXALATED VENOUS BLOOD	CAPILLARY BLOOD
1	20.0-19.0	19.5-19.5
2	14.5-14.0	14.0-14.0
3	17.5-16.0	18.5-18.0
4	15.0-16.0	16.0-15.0
5	18.0-18.5	17.0-16.0
6	15.0-16.0	15.5-14.5
7	17.0-16.0	17.0-17.5
8	17.0-17.0	19.0-19.0
9	16.0-16.0	17.0-18.0
10	17.0-17.0	18.0-18.5

timing of the test is from the time of the initial mixing of the blood with inverting the tube to this end point of a definite and easily visible precipitate. Table I tabulates the results of the comparisons of the Link-Shapiro modification of the Quick test for prothrombin time, using a plasma substrate, as compared to the capillary test tube technique using both oxalated venous and capillary blood samples on the same patients. The mean value for the prothrombin times of each technique is approximately 18 seconds. The variations within each test group, as determined by the standard deviation from the mean are equally and surprisingly similar, considering the usual range of error anticipated in biologic tests.

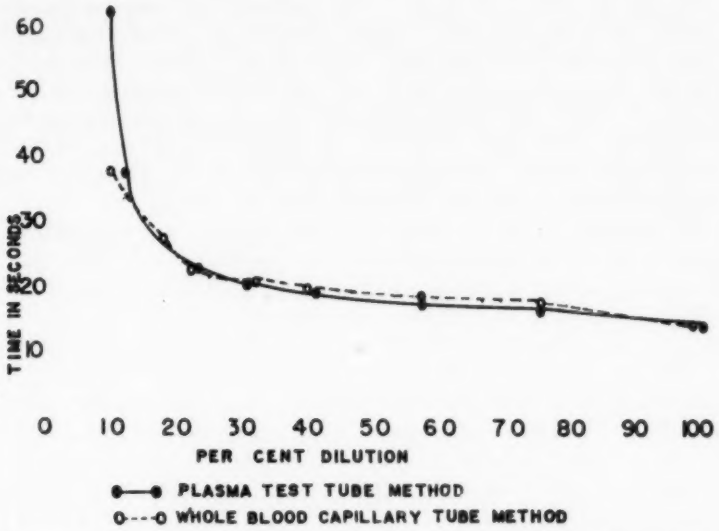

Fig. 1.—A comparison of prothrombin time dilution curves between the Link-Shapiro modification of the Quick test using plasma and the capillary tube method using oxalated venous blood.

RESULTS

Since this testing is done at room temperature rather than in the usual environmental temperature of 37° C., the variation of the capillary test tube technique at several different temperatures was investigated and is reported in Table II. The same sample of oxalated venous blood was used for each determination. First, a dilution curve using the modified Quick test on a plasma substrate at 37° C. was determined. Next, the capillary test tube method was used with the oxalated whole blood as a substrate maintaining the temperature of the blood and substrates at 37° C., 30° C., and finally at room temperature of 22° C. Reference to Table III confirms the accuracy of the capillary test tube technique with good correlation at all temperature ranges for the concentrated specimens to the 24 per cent dilution. From this point to the 10 per cent dilution, there is a larger margin of variation than would be permissible for the 22° C., but less so and in an acceptable range for the 30° and 37° C. runs. Fig. 1 describes a demonstration using a different

oxalated blood specimen. This figure compares the modified Quick test on the plasma substrates run at 37° C., while the capillary test tube technique was run at 22° C. The curves are very similar, with the tendency to lose accuracy only at the highest dilutions.

DISCUSSION

The major source of error in this capillary test tube technique lies in the need for a *free flow* of capillary blood. Any modicum of squeezing or local trauma that may be used to speed the flow of blood from the puncture site, no matter how trivial it may seem, creates marked variations of the prothrombin time in both directions. If the flow of blood from the puncture wound is too slow to be of use, a new site should be punctured for the determination rather than to attempt any manipulation whatsoever, no matter how gentle it would seem. This test, therefore, demands special familiarization with this aspect. The skill in recognition of the precipitant end point is quickly obtained in use and does not present a problem.

The convenience and accuracy of this capillary test tube technique for the determination of the prothrombin time using capillary blood has offered us a very useful tool to investigate prothrombin times in any special situation where adequate quantities of freely flowing venous blood cannot be obtained. This test is *not* recommended to supplant the usual techniques of prothrombin time determinations when they are convenient to do. We have had great success in using this test in the newborn nurseries for a specific research purpose and have used it in adults as well with equally satisfactory results (Table III). The bedside accuracy of this method plus the easy portability of the minimum equipment needed makes it potentially of great service for the speedy determination of prothrombin times in the hospital clinic or for home bedside use. The increasing use of long-term antiprothrombin drugs for anticoagulation therapy may require the use of such an inexpensive relatively accurate technique that can be used without requiring centrifugation or incubation of the samples, yet give an exact record of the blood prothrombin content.[6] The apparent simplicity of the test, however, should not minimize the very close attention to the important details discussed above.

SUMMARY

A bedside micromethod for the testing of prothrombin times using capillary blood as a substrate in a capillary glass test tube system at room temperature is described. This technique has been compared statistically with the usual Link-Shapiro modification of the Quick test in newborn infants as well as adults and found as accurate.

REFERENCES

1. Goldfader, A., Bloom, D., and Weiner, M.: A New Improved Method for the Determination of Prothrombin Levels in Blood, Science **111**: 365, 1950.
2. Isenberg, H. D.: A Micromethod for Prothrombin Time Determination, J. LAB. & CLIN. MED. **37**: 807-809, 1951.

3. Kato, K.: Micro-Prothrombin Test With Capillary Whole Blood, Am. J. Clin. Path. **10**: 147-153, 1940.
4. Dyggve, H.: Micro-method for the Quantitative Determination of Plasma Prothrombin and Proconvertin, Acta. med. scandinav. **150**: 207-214, 1954.
5. Rosling E., and Schoenheyder, E.: Micro-method for Owren's Prothrombin Determination, Scandinav. J. Clin. & Lab. Invest. **7** (Supp 14-22): 1955.
6. Manchester, B.: The Value of Continuous (1 to 10 Years) Long-Term Anticoagulant Therapy, Ann. Int. Med. **47**: 1202-1209, 1957.
7. Lewis, J. H., Ferguson, J. H., Spaugh, E., Fresh, J. W., and Zucker, M. D.: Acquired Hypoprothrombinemia, Blood **12**: 84-90, 1957.
8. Lewis, D. W., Munro, F. L., and Munro, M. P.: Prothrombin Determinations in Dicumarolized Patients: A Comparison of the One Stage and Bedside Methods, J. Lab. & Clin. Med. **35**: 16-23, 1950.
9. Willi, H.: New Micromethod for Determination of Prothrombin and Factor VII in the Newborn, Bibliot. paediat. **58**: 589-597, 1954.

A SEMIMICROTECHNIQUE FOR IRON^{59} BETA DETERMINATIONS IN BIOLOGIC MATERIALS

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IRON^{59} has been employed at the Radiobiological Laboratory of the University of Texas and the United States Air Force to evaluate iron incorporation into maturing erythrocytes of the monkey when the iron was administered in various forms,[1] and as an index of erythropoietic damage in monkeys exposed to mixed ionizing radiation.[2] In these studies, a modification of the method of Hennessy and Huff,[3] which utilizes crystal scintillator counting of the Fe^{59} gamma emissions, has been employed.

In hematologically abnormal or irradiated animals, erythropoietic activity may be markedly reduced or absent. To effect good counting under such conditions, it has sometimes been necessary to use 3.0 ml. of blood. In small animals, both sample volume and sampling frequency may be limiting.

The mode of decay of Fe^{59} to Co^{59} is by paired beta-gamma emission with mean energies of 1.1 mev. (gamma) and 0.46 mev. (beta), or 1.3 mev. (gamma) and 0.26 mev. (beta).[4] Consequently, the 1:1 beta-gamma ratio permits the use of either type of emanation in detection. Thin window or gas flow counting has been used to detect iron^{59} beta radiation,[5, 6] although recent work has usually employed gamma counting techniques in which crystal scintillators are used.[7, 8] Both techniques have inherent limitations; sample absorption can be limiting with the low-energy betas, while crystal scintillator absorption of relatively high-energy gammas frequently results in reduced counting efficiency.

This paper describes a technique for digesting iron-containing components of biologic systems, reducing and complexing the iron, and selectively extracting and concentrating the complex. In this form, total iron may be measured spectrophotometrically and then introduced into a liquid scintillator solution which permits beta counting of any Fe^{59} present.

MATERIALS AND METHODS

The care, maintenance, and clinical history of animals at this laboratory have been described.[9-12] Methods of preparing specific radioiron compounds, injection and counting techniques, and typical iron incorporation curves of these monkeys have also been reported in detail.[1, 2]

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This work was conducted at the Radiobiological Laboratory of the University of Texas and the United States Air Force, Austin, Texas, and was supported with funds provided under Contract AF 41(657)-149 and the School of Aviation Medicine.

Received for publication June 19, 1958.

To retain comparability with prior studies, two male *Macaca mulatta* monkeys analogous in age, weight, and clinical history to animals used in cited references were employed in this study.

A 1.0 ml. blood sample previously reserved for hematologic determinations was used for determining radioiron incorporation by the beta counting method under test, while the remainder was counted by the crystal scintillation method. This procedure tested replication of gamma counting data against the extensive previous work, and determined the ability of the beta technique to measure the identical phenomenon.

Beta Counting of Fe^{59} in a Liquid Scintillator System.—The procedure for digesting iron from protein complexes in blood, and selectively extracting it in recomplexed form, is a modification of the basic technique of Peterson.[14]

Either whole blood or subfractions may be used. The technique permits spectrophotometric determination of total iron. In addition, the same solution may be introduced into liquid scintillator solutions through secondary and tertiary solvent systems. In the tertiary solvent system, any iron^{59} present may be detected by counting photons resulting from the beta emanations.

Preparation of Whole Blood for Beta Counting.—

A. Reagents:

1. Concentrated sulfuric acid, reagent grade.
2. Trichloroacetic acid (TCA), reagent grade, 25 per cent solution in deionized water.
3. Sodium acetate, reagent grade, saturated solution in deionized water. Maintain excess sodium acetate in bottom of flask.
4. Thioglycollic acid, reagent grade.
5. 1,10-Phenanthroline (orthophenanthroline),* 0.5 per cent solution in isoamyl alcohol (IAA).†
6. Toluene.‡
7. Absolute ethanol (petroleum-derived for low C^{14} content).
8. Stock scintillation solution: 2,5-diphenyloxazole (5.45 Gm.) and 2,2′-p-phenylene-bis(5-phenyloxazole) (0.18 Gm.) in 1 liter of toluene.

B. Procedure:

1. Place 1.0 ml. concentrated sulfuric acid and a glass bead into a 50 ml. Pyrex centrifuge tube, and add 0.2 ml. whole blood. Under an exhaust hood, heat the mixture over a microburner until heavy white fumes appear. After 5 minutes, turn off the burner, and cautiously add 5.0 ml. deionized water while gently shaking the reaction tube. Allow the reaction mixture to cool to room temperature.

2. Add 0.1 ml. thioglycollic acid to the reaction mixture.

3. Add 2.0 ml. 25 per cent TCA to the reaction mixture and allow the precipitate to settle.

4. Add 10.0 ml. saturated sodium acetate (final pH should approximate 5.0) and centrifuge for 10 minutes at about 825 g.

5. Decant the clear supernatant into a clean 25 ml. glass-stoppered graduate cylinder. Add about 5 ml. IAA, stopper the cylinder, shake vigorously, and discard the IAA layer. This step removes IAA-soluble quenching agents from the system.

6. Add 5.0 ml. of the 0.5 per cent orthophenanthroline-IAA solution. Stopper and shake as in Step 5. This IAA layer, containing the red-orange iron-orthophenanthroline complex, may be transferred by capillary pipette.

7. If measurement of total iron is desired, this solution may be read spectrophotometrically at 512 mμ and compared to a standard curve. If no spectrophotometric determination is required, 2.0 ml. of the solution may be transferred directly to a Wheaton 5-dram Crystal Lite counting vial with polyethylene cap.

*G. Frederick Smith Chemical Company.
†Baker Chemical Company.
‡Phillips Petroleum Company.

8. To the IAA-iron-orthophenanthroline in the vial, add 7.0 ml. of the low C^{14} absolute ethanol. Then add 11.0 ml. of stock scintillation solution to bring the total volume to 20.0 ml.

9. Flush vials with argon for 3 to 5 minutes to remove oxygen, cap vials, and seal tops with collodion. Allow samples to attain photo and temperature equilibrium at -5° C.

Required Samples.—

A. Background sample: (1) Two milliliters of 0.5 per cent orthophenanthroline in IAA. (2) Seven milliliters of the low C^{14} absolute ethanol. (3) Eleven milliliters of the stock scintillation solution.

B. Injection standard: (1) Two-tenths milliliter of the incubated blood-isotope mixture, processed as any blood sample; however, due to high activity, use only 0.5 ml. of the iron-orthophenanthroline-IAA solution for counting. (2) Eight and one-half milliliters of the low C^{14} absolute ethanol. (3) Eleven milliliters of the stock scintillation solution. (This difference in concentrations of components has been found to be of no consequence.)

C. Incorporation samples (daily uptake): Two-tenths milliliter of whole blood processed as described.

D. Counting equipment and conditions:

1. Counter: Tri-Carb liquid scintillation spectrometer.*

2. Counter Settings:

High voltage tap	6
A and B circuit attenuation	1
A and B phototube voltage	1050
Coincidence counting setting (pos 3)	
Lower gate (both phototubes)	6.5 v.
Upper gate (both phototubes)	70.0 v.
Refrigerator temperature	-5° C.

3. External Factors:

Room temperature	26 ± 2° C.
Line voltage	120 ± 2 v.

E. Calculations:

1. Assumptions: (a) Half-life of Fe^{59} = 45.9 days (average).[4] (b) Blood volume of healthy monkey = 60.9 ml. blood per kilogram of body weight.[15]

2. Sample calculation:

(a) $\frac{\text{Volume of blood-Fe}^{59}\text{ mixture injected}}{\text{Volume used in preparing counting standard}} \times \text{counts per minute of standard} = \text{counts per minute injected.}$

(b) 0.2 ml. blood × 0.4 (two-fifths of IAA extract) = 0.08 ml. (0.08 ml. = volume of aliquot being counted).

(c) $\frac{\text{Net counts per minute in aliquot}}{0.08} \times \text{blood volume of animal} = \text{counts per minute in circulation.}$

(d) $\frac{\text{Amount of radioiron in circulation (c.p.m.)}}{\text{Amount of radioiron injected (c.p.m.)}} \times 100 = \text{per cent incorporation.}$

RESULTS

The iron-orthophenanthroline complex is colored. It consequently presents the possibility that photon absorption by the complex could be limiting. A spectrum of model systems employing known amounts of radioiron, both in colorless and a gradient of colored solutions, have been investigated and will be reported separately. Results indicate that, at concentrations of iron comparable to those derived from healthy monkeys, absorption does not limit the utility of the system.

*Packard Instrument Co., La Grange, Ill.

Absorption spectra of all components of the liquid counting system were determined on the Beckman D.U. spectrometer, separately and in all possible combinations. These spectra were then compared to the emission spectra of the 2,5-diphenyloxazole and 2,2′-*p*-phenylene-bis(5-phenyloxazole) as given by Hayes and associates.[16] Absorption and emission spectra evidenced a "tail" overlap in the region of 500 mμ which confirmed the observations made on model systems.

Initial beta spectrum (photon yield) determinations accomplished on the Tri-Carb liquid scintillation spectrometer evidenced a marked "shift" phenomenon characterized by erratic count rates from the same sample. However, when the sample was flushed with argon[13] and adequately sealed, the relative photon yield for the sample was stabilized. This apparent oxygen effect—now known to be attributable to oxidizable impurities sometimes found in two of the reagents—requires that samples be counted within about 6 hours after flushing and sealing.

Under the stipulated counter conditions, coincidence counting efficiency of blood-derived Fe^{59} consistently ranges from 35 to 40 per cent when intercompared with N.B.S. $Fe^{59}Cl_3$ secondary standards. Because the iron is homogeneously distributed in the counting system, and because of its intercomparability with the N.B.S. standard, the system may be regarded as an "absolute" beta counting technique.

Iron^{59} Incorporation.—Incorporation curves determined by both the beta and gamma counting techniques are shown in Fig. 1. The gamma counting data (obtained using the Nancy Wood crystal scintillation counter, Model SC-2L, and the calculations of Hennessy and Huff) reflect individual incorporation curves which are closely comparable in both rate and amplitude to individual animals of earlier studies.[1, 2] For illustrative purposes, the average value has been plotted.

TABLE I. NET COUNTS PER MINUTE PER MILLILTER OF BLOOD AS DETERMINED BY BOTH BETA AND GAMMA COUNTING TECHNIQUES

	NANCY WOOD CRYSTAL SCINTILLATOR* (MODEL SC-2L) ANIMAL NUMBER		TRI-CARB† (PACKARD LIQUID SCINTILLATION SPECTROMETER) ANIMAL NUMBER	
DAY	625	872	625	872
0 Injection assay	6,528	6,315	19,873	20,000
1	741	344	1,213	1,663
2	991	1,637	4,775	5,063
4	2,394	3,778	10,013	11,150
7	3,744	4,825	12,375	12,363
9	3,680	4,530	12,088	14,138
11	3,678	4,730	11,450	13,788
14	3,645	4,937	11,289	14,340

*Accomplished on blood volume of 1.0 ml. or greater.
†Accomplished on the equivalent of 0.08 ml. of blood.

Table I shows the net counts per minute per milliliter whole blood for each animal as determined by the beta and gamma counting techniques. These data were accrued prior to learning that quenching agents from the blood (lipids?) were being carried into the counting system. Extraction of these

materials prior to step 6 allowed blood-derived systems to be equated with model systems. Since the incorporation data in Fig. 1 were calculated from the proffered data, Table I very probably underrates the power of the beta counting method. Only the counting efficiency appears to be affected by the presence of the quenching materials.

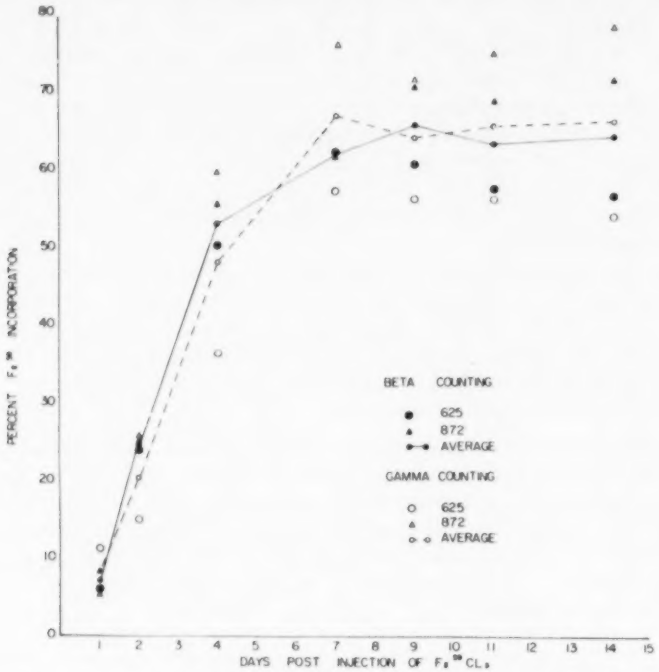

Fig. 1.—Comparison of iron[59] incorporation curves as determined by both beta and gamma counting methods.

DISCUSSION

The liquid scintillation method of counting Fe^{59} beta emissions permits reduction in blood sample volume when radioiron incorporation is used as an index of erythropoietic activity. Reductions in initial sample size range from $\frac{1}{5}$ (1.0 to 0.2 ml.) up to $\frac{1}{15}$ (from about 3.0 to 0.2 ml. whole blood).

The technique is capable of reproducing the incorporation patterns obtained by the commonly used gamma counting technique. Although only 2 test animals were used in this evaluation, the resultant data are in excellent agreement with both the concurrent gamma data and the extensive previous work. Additionally, it is apparent that there is less divergence between beta counts from the individual animals than is exhibited by the individual gamma counts. Consequently, a greater resolution is suggested which is probably attributable to both the increased counting efficiency of the technique and the greater precision inherent in coincidence counting.

Efficiency of the beta counting technique is from 35 to 40 per cent in blood-derived systems. Low level crystal scintillator counting of the associated 1.1 and 1.3 mev. gammas approximates an efficiency of 7 per cent in this laboratory. Net increase in efficiency is, therefore, from five- to sixfold. The beta counting technique for Fe^{59} was developed specifically for use with small laboratory animals in which blood sample volume and frequency may become limiting and was tested on apparently normal animals. If extremely low incorporation of the iron causes counting difficulties, the total iron in the counting system may be increased by adding more than 2.0 ml. of the iron-orthophenanthroline complex (in the IAA extract) to the counting system with a concomitant reduction in the amount of ethanol used. However, the efficiency of counting must decline as concentration of the complex in the counting solution is increased; fall off in efficiency approximates linearity over the concentration range thus far investigated.

The technique suggests potential clinical application in cases where infants and small children are under study for blood dyscrasias, etc. When coupled with spectrophotometric determinations of total iron in blood, its subfractions, or in other biologic components, the beta counting technique lends itself to rapid calculation of specific activity which is required in the study of ferrodynamics.

SUMMARY

After acid digestion of iron-containing components of biologic systems, the iron may be selectively complexed and extracted from the digest. The colored complex is of adequate stability to permit spectrophotometric measurement of total iron. If $iron^{59}$ is also present in the system, the same colored complex may be introduced into a liquid scintillator solution and the beta emanations counted with an efficiency of from 35 to 40 per cent at the concentrations of iron derived from blood. The technique offers a net increase in counting efficiency of from five- to sixfold as compared to the commonly used gamma counting technique, a reduction in size of the initial sample up to one-fifteenth, and a substantial, but unquantitated, increase in the precision of counting.

REFERENCES

1. Hartwig, Q. L., Leffingwell, T. P., Young, R. J., and Melville, G. S., Jr.: Comparative Erythropoietic Incorporation of Ferric and Ferrous Preparations of $Iron^{59}$ by the Macaca mulatta Monkey, J. Lab. & Clin. Med. **51**: 410-414, 1958.
2. Hartwig, Q. L., Melville, G. S., Jr., Leffingwell, T. P., and Young, R. J.: $Iron^{59}$ Metabolism as an Index of Hematopoietic Damage and Recovery in Monkeys Exposed to Nuclear Radiations, School of Aviation Medicine, United States Air Force, Report No. 58-59.
3. Hennessy, T. G., and Huff, R. L.: Depression of Iron Uptake in X-Irradiated Rats, Proc. Soc. Exper. Biol. & Med. **73**: 436-440, 1950.
4. Radiological Health Handbook, United States Department of Health, Education, and Welfare, 1955.
5. Redishe, J. H., Palmer, R. F., and Cline, J. F.: Assay of $Iron^{59}$ and $Iron^{55}$ in Biological Samples, Anal. Chem. **27**: 849-850, 1955.
6. Wasserman, L. R.: Rate of Removal of Radioiron From the Plasma as an Index of Erythropoiesis, J. Clin. Invest. **31**: 32-39, 1952.
7. Huff, R. L., Elmlinger, P. J., Garcia, J. F., Oda, J. M., Cockwell, M. C., and Lawrence, J. H.: Ferrokinetics in Normal Persons and in Patients Having Various Erythropoietic Disorders, J. Clin. Invest. **3**: 1512-1526, 1951.

8. West, H. D., Hahn, P. F., Clark, W. F., and Chappele, E. W.: Labelled Iron Stores in Study of Iron Liberated During Red Cell Destruction by Acetylphenylhydrazine, Am. J. Physiol. **169**: 194-197, 1952.
9. Kent, S. P., Vawter, G. F., Dowben, R. M., and Benson, R. E.: Hypervitaminosis D in Monkeys: A Clinical and Pathologic Study, Am. J. Path. **34**: 37-60, 1958.
10. Krise, G. M., Jr., and Wald, N.: The Normal Blood Picture of the Macaca mulatta Monkey, J. Appl. Physiol. (In press.)
11. Melville, G. S., Jr., Hartwig, Q. L., Leffingwell, T. P., and Young, R. J.: Evidences of Hematologic Recovery in Monkeys Exposed to Hypervitaminosis D. School of Aviation Medicine, United States Air Force, Report No. 58-57.
12. Young, R. J., Fremming, B. D., and Harris, M. D.: Care and Management of a Macaca mulatta Monkey Colony, Proc. Animal Care Panel **7**: 2, 1957.
13. Ott, D. G., Hayes, F. N., Hammel, J. E., and Kephart, J.: Argon Treatment of Liquid Scintillators to Eliminate Oxygen Quenching, Los Alamos Scientific Laboratory, Report No. 1837, October, 1954.
14. Peterson, R. E.: Determination of Serum Iron Using 4,7-diphenyl-1,10-phenanthroline, Anal. Chem. **25**: 1337-1339, 1953.
15. Bender, M. A.: Blood Volume in the Rhesus Monkey, Science **122**: 156, 1955.
16. Hayes, F. N., Rogers, B. S., Sanders, P., Schuch, R. L., and Williams, D. L.: Liquid Solution Scintillators, Los Alamos Scientific Laboratory Report No. 1639, October, 1953.

THE DETERMINATION OF FREE AND TOTAL CHOLINE IN SERUM AND ITS APPLICATION TO THE PARTITION OF SERUM PHOSPHOLIPIDS

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THIS paper deals with three problems: (1) the validity of the microbiologic assay of total choline in serum; (2) the measurement of free choline in serum; and (3) the partition of serum phospholipids into those containing choline and those that do not.

Bligh[1] and Appleton and associates[2] believe the microbiologic assay originally described by Horowitz and Beadle[3] and used by Luecke and Pearson[4] for the measurement of the choline content of plasma is not specific for choline. However, Duell[5] believes this method is quite sensitive and specific. Review of the original articles by Horowitz and Beadle[3] and Luecke and Pearson[4] indicate several studies were omitted that are necessary to establish the validity of this type of assay. The results of experiments designed to test the validity of the microbiologic assay are reported here and indicate the measurement of *total* choline in serum is specific, accurate, and reproducible.

The measurement of *free* choline in serum was also undertaken. Bligh[1] has summarized beautifully the problems inherent in the measurement of free choline in serum. Using a biologic method he found plasma contained 1 to 2 μg choline per milliliter. Appleton and co-workers[2] measuring a periodide precipitate of choline in ultraviolet light found an average of 4.4 μg choline per milliliter of plasma. These are in contrast to the findings of Luecke and Pearson[4] of 44 to 75 μg choline per milliliter of plasma using the microbiologic method. The results presented below indicate that contrary to the findings of Luecke and Pearson[4] there is no detectable *free* choline in serum using the microbiologic method.

There is also disagreement about the quantity of choline-containing and noncholine-containing phospholipids in human plasma. Earlier work, summarized by Taurog and associates[6] indicated 20 to 40 per cent of serum phospholipids are noncholine containing. Albrink[20] more recently has confirmed this earlier work. However, Taurog and associates[6] found that only 0 to 8 per cent of serum phospholipids do not contain choline. These findings were confirmed by Hack[7] and Sinclair,[8] although review of their data indicates that in some of the plasma samples examined as much as 8 to 17 per cent of the serum phospholipids are free of choline. Our studies to date in 38 normal

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This study was supported in part by grants from the American Heart Association and United States Public Health Service.

Received for publication July 15, 1958.

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males, aged 20 to 56 years, indicate that 10 to 31 per cent of the serum phospholipids do not contain choline, this being in substantial agreement with the findings of Albrink.[20]

METHODS

The essentials of this microbiologic method are the measurement of the growth response of a choline dependent mold in a growth media supplemented with choline present in hydrolysates, or acetone extracts, of serum. The serum hydrolysates for the total choline determination, and the acetone extracts of serum for free choline determination are prepared according to the methods of Luecke and Pearson.[4] The hydrolysates of serum were sealed and sterilized in Pyrex tubes and stored until used. The acetone extracts of serum were prepared just before use.

The growth response of the mold to the test solutions were measured by the method originally described by Horowitz and Beadle[3] except 3 different aliquots of test material were analyzed in duplicate simultaneously.

Chromatographic columns of the type originally described by Dubnoff and Borsork[9] and used by Horowitz and Beadle[3] and Luecke and Pearson[4] were filled with wet Permutit* (0.5 to 1.0 Gm.) previously activated by mixing with distilled water for 10 minutes. These columns were used as necessary to separate choline from the hydrolysates or the acetone extracts. The adsorbed choline was washed with 5 ml. 0.3 per cent NaCl and eluted with 10 ml. of near boiling 10 per cent NaCl. Columns of the type described were used to emulate as closely as possible the method originally described by Luecke and Pearson.[4]

Lipid phosphorus determinations were made by the method of Fiske and Subbarow,[10] using certain modifications in volumes, and 2,4-amino-napthal-sulfuric acid for color development.

TABLE I. RECOVERY OF CHOLINE CHLORIDE ADDED TO SERUM

SERUM	SERUM CHOLINE	ADDED CHOLINE	TOTAL CHOLINE PRESENT	TOTAL CHOLINE RECOVERED	PER CENT CHOLINE RECOVERED
Serum Hydrolysates Passed Through Column of Decalso					
A	385*	58	443	438	98.8
B	269	116	385	363	94.3
C	425	232	657	626	95.4
D	316	289	605	665	109.0
Serum Hydrolysates Assayed Directly					
E	306	289	595	606	101.8
F	356	116	472	486	103.0
G	307	289	596	594	99.5
H	301	58	359	364	101.0
I	463	231	694	725	104.0
J	463	116	579	605	104.0
K	463	58	521	557	107.0

*The values are expressed in micrograms of choline chloride per milliliter of serum.

RESULTS

I. *The Validity of the Microbiologic Assay of Choline.*—

Recovery experiments: Recovery of choline added to serum as choline chloride or as purified egg lecithin is complete, as shown in Tables I and II. The total choline in serum whether initially free or bound in phospholipids is therefore quantitatively measured.

*Decalso, obtained from The Permutit Company, New York, N. Y.

TABLE II. LIBERATION OF CHOLINE FROM LECITHIN* BY ACID HYDROLYSIS

SERUM	SERUM CHOLINE	CHOLINE ADDED AS LECITHIN	TOTAL CHOLINE PRESENT	TOTAL CHOLINE RECOVERED	PER CENT CHOLINE RECOVERED
A	394†	195	589	567	96.4
B	447	195	642	659	102.0
C	394	3,270	3,664	3,264	89.1

*A known amount of lecithin was added to serum samples and the choline content was determined as described under Methods. Pure lecithin was prepared from egg yolk according to the method of Levene and Rolf.[13] The pure lecithin contained: phosphorus 3.79 per cent, carbon 63.18 per cent, hydrogen 10.21 per cent, nitrogen 1.70 per cent, and choline 14.0 per cent. The choline-phosphorus molar ratio equaled 0.89, and that of nitrogen-phosphorus equaled 0.99. The theoretical values of the elements, choline, and the molar ratios are 4.1 per cent, 64.8 per cent, 10.8 per cent, 1.80 per cent, 15.3 per cent, 1.0, and 1.0.

†The values are expressed in micrograms of choline chloride per milliliter of serum.

Chromatographic purification: Recoveries of standard solutions of choline chloride averaging 99±4 per cent were obtained with columns of Permutit prepared as described under Methods. Dry Permutit or Permutit activated as described by Hennessey[13] and used by Luecke and Pearson[4] gave only 80 per cent recoveries in our hands.

The results of passing serum hydrolysates through the columns of Permutit are summarized in Tables I and III. It is seen the absorption procedure does not alter the mean value or enhance the recovery of serum choline and, therefore, is unnecessary.

TABLE III. THE RESULT OF PASSING SERUM HYDROLYSATES THROUGH A COLUMN OF PERMUTIT

SERUM	MEAN	
	UNTREATED	TREATED
A	308*	287
B	306	345
C	594	665
D	368	365
E	375	377
F	312	322
G	384	379
H	347	349
I	280	305
J	284	311
Mean of Means ± S.E.	356 ± 29.1	370 ± 34.3

$$\text{S.E.} = \frac{\text{Standard Deviation}}{\sqrt{\text{n-1}}}$$

$$t = 0.31$$

*Choline chloride concentration expressed in micrograms per milliliter of serum.

Comparison with the reineckate procedure: Table IV compares serum choline values obtained with reineckate colorimetric method[14] and the microbiologic method. The mean values are in excellent agreement and indicate that the microbiologic assay of choline is comparable to that of a generally accepted chemical method.

Specificity of the assay: No attempt was made to confirm the lack of effect on the growth of the mold of the many compounds tested by other authors.[3, 15] However, methionine and lecithin growth activities were determined and found to be 0.004 and 0.5 times as active as choline, respectively. Lecithin

should not interfere with the assay as it is completely hydrolyzed under the conditions used in these experiments. Also, methionine is not present in serum in sufficient amounts to act as a choline substitute.

TABLE IV. COMPARATIVE CHOLINE ANALYSES OF BLOOD SERUM

SERUM	ASSAY METHOD	
	REINECKATE (MG.%)	MICROBIOLOGIC (MG.%)
A	42.0 ± 1.0*	42.5 ± 0.0*
B	30.0 ± 2.7	30.1 ± 0.5
C	35.3 ± 0.2	36.4 ± 1.0
D	31.2 ± 0.5	32.7 ± 0.3
E	28.6 ± 2.5	26.9 ± 0.5
F	32.3 ± 3.0	33.2 ± 4.0
G	31.5 ± 0.0	31.3 ± 0.1
H	43.7 ± 0.2	42.7 ± 0.9
I	39.0 ± 1.5	35.9 ± 0.1

*Average of duplicate assays.

Serum choline concentrations and assay variation: The variation of replicates about a mean assay value is independent of the choline concentrations. This was demonstrated by comparing the coefficients of variation of assays determined over a wide range of serum choline concentration. The mean coefficients of variation are comparable, regardless of choline concentration, and both the mean coefficient of variation and the range of such coefficients are less than the corresponding variations on the repeated assays of control material and serum. Table V summarizes these data.

TABLE V. LACK OF INFLUENCE OF SERUM CHOLINE CONCENTRATION ON THE COEFFICIENTS OF VARIATION OF MICROBIOLOGIC VALUES

RANGE OF SERUM CHOLINE CONCENTRATION	NUMBER OF SAMPLES ASSAYED	MEAN C.V.* OF SAMPLES ANALYZED	RANGE OF C.V. OF SAMPLES ANALYZED
200-249	2	5.77	4.22-7.32
250-299	6	5.87	4.54-7.47
300-349	6	4.98	3.79-6.71
350-399	6	3.44	0.85-4.74
400-449	6	4.11	3.39-4.74
450-499	6	3.69	1.25-5.30
Repeated serum analyses	7	5.93	3.03-9.11
Repeated control analyses	23	6.30	2.63-10.03

*C.V. = coefficient of variation = $\sigma/m \times 100$; σ = standard deviation; m = mean of 6 replicates.

Reproducibility of assays: It is necessary to evaluate the reliability of the standard growth response curve in each experiment. This was done by assaying a standard material in all experiments. A commercial lecithin product* was dissolved in chloroform and then treated exactly as are the sera. This material was assayed many times over a 3-month period and 23 unselected assays were analyzed statistically. According to the F-test[12] the chances are less than 1 in 100 that the assay results are not homogenous. The standard

*Lecithin Pure, obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

error of the means of repeated assays of this lecithin control material is ±0.72 per cent, so that the 95 per cent confidence limits for such a mean are ±1.96 times the standard error or ±1.41 per cent. Likewise the standard error of the means of 7 assays of the same serum analyzed in 7 different experiments is ±2.39 per cent so that the 95 per cent confidence limits for such a mean are ±1.96 times 2.39 per cent, or ±4.64 per cent. The above calculations indicate that assay results are reproducible and, as concerns serum, with an accuracy of ±4.64 per cent.

II. *The Analysis of Free Choline in Serum.*—The studies above reveal that the *total* choline content of serum can be measured accurately and reproducibly. Similar quantitative data could not be obtained in testing for *free* choline in serum. Sera were analyzed for their free choline content using the method of Luecke and Pearson.[4] Contrary to their findings, the growth response of the mutant to different amounts of the same extract were not parallel and usually showed a marked "drift." This suggested accessory growth substances in addition to free choline were being measured. To remove these the serum extract was passed through a column of Permutit, then washed and eluted in the usual manner. Subsequent to such treatment the presence of an "active" growth substance could not be demonstrated in the filtrate, wash, or eluate. Whatever substances were active initially were completely removed by the adsorbent and were impossible to recover in the usual way.

Since the serum extract is a cloudy mixture, the free choline might have been adsorbed to some particulate matter and the forces binding this hypothetic complex to the adsorbent might not be disruptable with the usual polar eluents. To test this possibility, a known amount of choline chloride was added to serum-acetone mixtures. These mixtures were allowed to stand several hours, were treated as before, and then passed through a column of Permutit. After such treatment only the added choline was recovered in the eluate. These studies indicate to us that free choline is not present in serum except possibly in exquisitely small amounts as indicated by Bligh[1] and Appleton and associates.[2]

III. *The Partition of Serum Phospholipids.*—Assuming that free choline is present in serum in trace amounts (1 to 2 μg/ml.),[1] if at all, we will not be in significant error if it is assumed that essentially all choline in serum is derived from the phospholipids, lecithin and sphingomyelin, and measurement of the total choline in serum is a measure of the choline-containing phospholipids, assuming one molecule of choline to each molecule of phospholipid. The molal ratio of choline to lipid phosphorus would then represent the fraction of total phospholipids that contain choline and 1.0 minus this fraction equals the amount of noncholine-containing phospholipid or cephalin.

Thirty-eight normal male sera were analyzed for lipid phosphorus and total choline. Phospholipid was calculated by multiplying the lipid phosphorus by 25. The choline-lipid phosphorus molal ratio was then calculated.

TABLE VI. THE CHOLINE-CONTAINING AND NONCHOLINE CONTAINING PHOSPHOLIPIDS OF NORMAL HUMAN SERUM

MEN	SAMPLE	DAYS BETWEEN ASSAYS	AGE	TOTAL LIPIDS* CHOLINE (MG.%)	TOTAL LIPIDS* P (MG.%)	CHOLINE / LIPID P†	PL (MG. %)	CCPL (MG. %)	NCCPL (MG. %)	CCPL (%)	NCCPL (%)
He	1	---	20	35.9	10.4	0.765	260	199	61	76.5	23.5
Co	2	---	22	36.4	10.0	0.808	250	202	48	80.8	19.2
Co	2a	8	22	32.4	8.79	0.819	219	179	40	81.9	18.1
Br	3	---	23	33.2	9.58	0.770	239	184	55	77.0	23.0
Hu	4	---	23	32.7	8.22	0.884	206	182	24	88.4	11.6
Bl	5	---	24	26.9	7.75	0.770	194	169	25	77.0	23.0
Mo	6	---	27	35.9	10.2	0.781	255	199	56	78.1	21.9
Se	7	---	28	35.1	9.87	0.790	247	195	52	79.0	21.0
Po	8	---	29	33.4	10.4	0.708	261	185	76	70.8	29.2
Po	8a	44	29	30.3	8.73	0.770	218	168	50	77.0	23.0
Ta	9	---	29	31.9	9.10	0.779	228	178	50	77.9	22.1
Ta	9a	60	29	37.1	9.68	0.850	242	205	37	85.0	15.0
Ta	9b	390	29	35.8	10.2	0.779	255	199	56	77.9	22.1
Ra	10	---	29	44.6	12.5	0.792	312	247	65	79.2	20.8
Ra	10a	60	29	54.2	15.1	0.797	377	300	77	79.7	20.3
Jo	11	---	32	29.5	7.8	0.839	195	164	31	83.9	16.1
Jo	11a	90	32	28.8	8.3	0.770	208	159	48	77.0	23.0
Jo	11b	270	32	31.3	9.94	0.699	248	173	75	69.9	30.1
De	12	---	32	30.1	9.25	0.721	231	166	65	72.1	27.9
De	12a	14	32	30.7	9.64	0.708	241	171	70	70.8	29.2
Sl	13	---	32	26.9	8.20	0.730	205	150	55	73.0	27.0
Le	14	---	32	35.6	9.77	0.808	244	197	47	80.8	19.2
Le	14a	14	32	37.2	10.1	0.817	252	206	46	81.7	18.3
Ma	15	---	33	33.2	9.25	0.794	231	183	48	79.4	20.6
Ma	15a	90	33	36.9	9.67	0.847	242	205	37	84.7	15.3
Pu	16	---	33	26.6	7.19	0.821	180	148	32	82.1	17.9
Pu	16a	5	33	38.8	8.54	0.748	214	160	54	74.8	25.2
Bla	17	---	33	30.6	8.71	0.779	218	170	48	77.9	22.1
Dew	18	---	33	28.5	8.25	0.768	206	158	48	76.8	23.2
Me	19	---	34	34.9	9.64	0.804	241	194	47	80.4	19.6
Me	19a	45	34	31.5	9.85	0.710	246	175	71	71.0	29.0
Go	20	---	34	27.9	8.66	0.714	216	154	62	71.4	28.6
Go	20a	7	34	31.6	9.02	0.777	226	176	50	77.7	22.3
Ho	21	---	34	34.8	9.31	0.830	232	193	39	83.0	17.0
Ho	21a	120	34	29.3	9.50	0.686	238	163	75	68.6	31.4
Sk	22	---	36	29.2	7.18	0.904	180	162	18	90.4	9.6
Kl	23	---	36	40.6	10.9	0.821	274	225	49	82.1	17.9
Kl	23a	30	36	36.6	10.3	0.788	258	203	55	78.8	21.2
Kl	23b	120	36	39.9	10.9	0.814	273	222	51	81.4	18.6
Kl	23c	180	36	34.0	10.5	0.719	263	189	74	71.9	28.1
Eb	24	---	36	36.1	10.0	0.801	250	200	50	80.1	19.9
Hag	25	---	38	34.9	10.2	0.759	255	194	61	75.9	24.1
Hag	25a	30	38	38.2	9.9	0.857	248	212	36	85.7	14.3
Cor	26	---	39	37.1	10.3	0.799	258	206	52	79.9	20.1
Cor	26a	7	39	44.7	11.8	0.841	295	248	47	84.1	15.9
Ev	27	---	40	46.7	13.5	0.768	336	256	80	76.8	23.2
Ev	27a	4	40	44.7	12.8	0.774	320	248	72	77.4	22.6
Fr	28	---	40	24.4	7.13	0.759	178	135	43	75.9	24.1
Ke	29	21	40	36.4	10.8	0.748	271	203	68	74.8	25.2
Ke	29a	---	40	46.0	12.3	0.830	308	256	52	83.0	17.0
Ga	30	---	41	39.0	10.4	0.832	260	216	44	83.2	16.8
Joh	31	---	46	40.8	11.7	0.777	292	227	65	77.7	22.3
St	32	---	47	39.2	11.3	0.777	282	217	65	77.0	23.0
St	32a	90	47	34.5	10.0	0.765	250	191	59	76.5	23.5
Da	33	---	51	35.4	9.74	0.806	242	195	47	80.6	19.4
Lu	34		51	37.5	11.6	0.719	290	209	81	71.9	28.1
Lu	34a	14	51	38.2	11.4	0.743	285	212	73	74.3	25.7
Hog	35	---	56	52.5	13.5	0.863	338	291	47	86.3	13.7
Mi	36	---	56	36.4	9.66	0.837	242	203	39	83.7	16.3
Mi	36a	7	56	35.7	10.0	0.792	255	202	53	79.2	20.8
Hus	37	---	38	35.7	9.3	0.852	233	199	34	85.2	14.8
Carl	38	---	80	39.9	10.7	0.828	267	221	46	82.8	17.2

*Choline values expressed as the chloride.

†Molal ratio M.W. P = 31, M.W. choline = 139.5.

Key to abbreviations: P = phosphorus; PL = phospholipids; CCPL = choline-containing phospholipids (lecithin and sphingomyelin); NCCPL = noncholine-containing phospholipid (cephalin).

The remainder of the calculations were made as follows:

$$\frac{\text{Choline}}{\text{Lipid P}} \times \text{Phospholipid (mg. per cent)} = \text{CCPL}^{*} \text{ mg. per cent}$$

$$\left(1.0 - \frac{\text{Choline}}{\text{Lipid P}}\right) \times \text{Phospholipid (mg. per cent)} = \text{NCCPL}^{\dagger} \text{ mg. per cent}$$

The results are summarized in Table VI and indicate CCPL in serum vary from 68.6 to 90.4 per cent and the NCCPL represents 9.6 to 31.4 per cent of the total phospholipids. Also apparent is the minimal variation from day to day and month to month in the phospholipid fractions of many of the subjects studied, suggesting that some mechanism controls the relative constancy of these fractions.

DISCUSSION

Chemical Methods.—To date chemical methods for choline determinations are nonspecific.[16] Recent modifications in the reineckate method[14] make this method desirable and it has been a useful tool in our hands but reproducible results demand the assay of at least 10 ml. of serum, which is prohibitive for routine use. This motivated our undertaking the microbiologic method.

The Microbiologic Method.—Gyorgi[16] has delineated the criteria for establishing the validity of a microbiologic method. We used these criteria to establish the quantitative limits of this method. It was found that (1) total choline is quantitatively recoverable whether present in a free or bound state; (2) assays are reproducible from day to day; (3) there exists no "drift" in the assay values when different volumes of hydrolysate are assayed; (4) an adsorption-elution chromatographic procedure does not enhance the accuracy of assay values, and substances present in the hydrolysates collectively do not alter the rate of choline utilization by the mold; (5) assay results are comparable to those obtained with a generally accepted method; (6) the assay is specific for choline; and (7) the coefficient of variation of the analysis is independent of the choline concentration in serum. The results indicate the microbiologic method for the assay of total choline in serum is quantitative and valid.

The microbiologic method proves to have several advantages. Sera can be collected and analyzed when convenient, as serum hydrolysates prepared and stored as described above are stable at room or refrigerator temperatures for at least 6 months. Also, a large number of serum hydrolysates can be analyzed during one assay procedure, the only factor limiting the number of assays being the size of the incubator. The average working time for each serum choline analysis exclusive of incubation time is about one hour. Also only small amounts of assay material are necessary. As little as 0.1 ml. of human and rat sera has been successfully assayed. Such small serum requirements permit the routine analysis of serum choline concurrently with other serum lipid and lipoprotein studies. Indeed this method could also be applied to the partition of the phospholipids of lipoproteins separated by ultracentrifugation.

*CCPL = choline-containing phospholipids (lecithin and sphingomyelin).
†NCCPL = noncholine-containing phospholipids (the cephalins).

Free Choline in Serum.—Luecke and Pearson[4] using the microbiologic method demonstrated that 44 to 75 μg choline per milliliter of plasma is free. This was not confirmed in our laboratory using serum. Our experiments revealed that acetone extracts of serum contain some accessory growth substance for the mold, but this is not free choline, and is probably acetone soluble lecithin as has been shown by Sinclair[8] and confirmed by Entenman and associates.[17] It might also be pointed out that Luecke and Pearson[18] used purified lecithin probably originally isolated by acetone precipitation to demonstrate the completeness of precipitation of phospholipids in serum treated with acetone. One cannot assume, however, native phospholipid will behave as does chemically pretreated phospholipid. Also Luecke and Pearson[4, 18] used only two different aliquots of assay material. Experience with microbiologic methods has shown that 3 different aliquots of assay material must be used to make evident the possible presence of accessory growth substances, that are not otherwise apparent when two aliquots are used.

Sources of Serum Choline.—Choline in serum is present in the phospholipids (lecithin and sphingomyelin), in acetylcholine, and free. Acetylcholine is present in but trace amounts[19] as is free choline.[1, 2] The choline-containing phospholipids appear to be the only significant source of serum choline, and for our purposes choline may be considered to be transported in serum primarily in the bound state. No evidence for the presence of free choline could be found by our methods.

The Partition of Serum Phospholipids.—There is still disagreement regarding the composition of serum phospholipids. Our studies indicate substantial amounts of serum phospholipids are not choline-containing in the normal human male, confirming the work of many authors cited by Taurog and associates.[6] It is believed that as larger numbers of human sera are analyzed for their choline- and noncholine-containing phospholipids even larger amounts of cephalin will be discovered in various plasma, and it is possible there exist some individuals with minimal amounts of choline-containing phospholipids.

Statistical Analysis and Validity of Assay Results.—Three different volumes of the same solution are assayed in duplicate, and each assay value is determined from the mean of 6 replicates. The mean and standard deviation of the mean of the 6 replicates are then calculated. If two or more of the replicates fall beyond two standard deviations the assay is rerun. If one of the replicates falls beyond two standard deviations, inclusion of this suspect value may or may not increase the accuracy of the mean. To determine if discarding the suspect value will result in a probable (odds of 3:2) increase in the accuracy of the mean the following test is applied.* Inclusion of the suspect value will increase by a certain percentage the range between the lowest and highest value. Subtracting the replicate value nearest the suspect replicate from the suspect replicate and dividing this difference by the difference between the highest and lowest replicate value exclusive of the suspect value and multiplying by 100 will give the range increase contributed by

*Derived by Dr. Allan Walker and Miss Lucille Hac of the Amino Products Division of the International Minerals and Chemicals Corporation.

the suspect replicate. If the per cent increase in the range was less than 87 per cent, then the suspect value was discarded with odds of 3 to 2 that there will result a probable increase in the accuracy of the mean.

It has been our experience that less than 10 per cent of all assays have to be repeated. The remaining 90 plus per cent are valid assays, and of these 80 per cent have been determined from the mean of 6 replicates and 20 per cent from the mean of 5 replicates.

SUMMARY

1. Experiments designed to test the validity of the microbiologic assay of choline in serum reveal that the total choline in serum can be quantitatively measured.

2. Free choline is not present in serum as herein measured.

3. Studies on 38 normal men, 20 to 56 years of age, indicate that 68 to 90 per cent of serum phospholipids are choline-containing (sphingomyelin and lecithin) and 10 to 32 per cent are noncholine-containing (the cephalins).

The helpful criticisms of Drs. Mary Lou Eilert, Robert Wissler, and Richard J. Jones are gratefully acknowledged.

REFERENCES

1. Bligh, J.: The Level of Free Choline in Plasma, J. Physiol. **117**: 234-240, 1952.
2. Appleton, H. A., LaDu, B. N., Jr., Levy, B. B., Steele, J. M., and Brodie, B. B.: A Chemical Method for the Determination of the Free Choline in Plasma, J. Biol. Chem. **205**: 803-813, 1953.
3. Horowitz, N. H., and Beadle, G. W.: A Microbiological Method for the Determination of Choline by Use of a Mutant of Neurospora, J. Biol. Chem. **150**: 325, 1943.
4. Luecke, R. W., and Pearson, P. B.: The Microbiological Determination of Free Choline in Plasma and Urine, J. Biol. Chem. **153**: 259, 1944.
5. Duell, H. J., Jr.: Lipids, New York, 1951, Interscience Publishers, Inc., vol. 1, p. 432.
6. Taurog, A., Entenman, C., and Chaikoff, I. L.: The Choline-Containing and Non-Choline Containing Phospholipids of Plasma, J. Biol. Chem. **156**: 385, 1944.
7. Hack, M. H.: Estimation of the Phospholipids in Human Blood, J. Biol. Chem. **169**: 137-143, 1947.
8. Sinclair, R. G.: The Lecithin, Cephalin and Sphingomyelin Content of Serum, J. Biol. Chem. **174**: 343-353, 1948.
9. Dubnoff, J. W., and Borsook, H.: A Micromethod for the Determination of Glycocyamine in Biological Fluids and Tissue Extracts, J. Biol. Chem. **138**: 381, 1941.
10. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375, 1925.
11. Levene, P. A., and Rolf, I. P.: The Preparation and Purification of Lecithin, Biochem. J. **72**: 587, 1927.
12. Snedecor, G. W.: Statistical Methods Applied to Experiments in Agriculture and Biology, ed. 4, Ames, Iowa, 1946, The Collegiate Press, Inc.
13. Hennessey, D. J.: Chemical Methods for the Determination of Vitamin B, Indust. & Engin. Chem. (Anal. Ed.) **13**: 216, 1941.
14. Glick, D.: Concerning the Reineckate Method for the Determination of Choline, J. Biol. Chem. **156**: 643, 1944.
15. Jukes, T. H., and Dornbush, A. C.: Growth Stimulation of Neurospora Cholineless Mutant of Dimethylaminoethanol, Proc. Soc. Exper. Biol. & Med. **58**: 142, 1945.
16. Gyorgi, P. (Editor): Vitamin Methods, New York, 1950, Academic Press, Inc., vols. I and II.
17. Entenman, C., and Chaikoff, I. L.: On the Determination of Choline in the Liver and Plasma of the Dog, J. Biol. Chem. **160**: 377, 1945.
18. Luecke, R. W., and Pearson, P. B.: The Determination of the Choline in Animal Tissues, J. Biol. Chem. **155**: 507, 1944.
19. Sollman, T.: Manual of Pharmacology, ed. 6, Philadelphia, 1942, W. B. Saunders Company, p. 390.
20. Albrink, M. J.: The Choline Containing Phospholipids of Serum, J. Clin. Invest. **29**: 46, 1950.

STERILIZATION OF PLASMA COMPONENTS BY HEAT

I. β_1-Metal-Combining Protein

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THE goal of this study has been to devise a means of preparing solutions of β_1-metal-combining protein which may be used clinically with minimal risk of transmitting infectious agents which may have been present in the plasma pool from which the globulin was prepared.

The clinical use of pooled plasma and fractions prepared from it has been seriously limited by the possibility of transmission of the virus of homologous serum hepatitis. Great effort has been expended in attempts to destroy the virus without damaging the plasma proteins at the same time.

Of all the proposed techniques, heating at 60° C. for 10 hours is recognized as the most dependable means of destroying the virus.[1, 2] Unfortunately, the properties of the plasma proteins may be greatly altered by this process. Stabilizers have been discovered for plasma albumin which permit solutions of this protein to be heated for 10 hours at 60° C. without appreciable alteration.[3-5] These stabilizers have also made possible the preparation of transfusion agents comprised of globulins and albumins which can be sterilized by the above-mentioned heating technique.[6-9] Conditions whereby the plasma enzyme precursor, plasminogen, can be similarly heated without important loss of its biologic activity have also been found.[10] With the exception of the above instances, successful heat sterilization of plasma components has not been described and clinical study of plasma constituents has been thereby immeasurably delayed.

The known stabilizers of plasma proteins have proved to be specific. Caprylate will stabilize the albumin of plasma but has no similar effect for the globulins. Glycine has increased the stability of solutions of gamma globulin but has no stabilizing effect on albumin. We have thus approached the problem of preparing plasma proteins for clinical study by working with at least partially purified components where one need consider only the properties of the particular protein in question without reference to effects of the procedure on other plasma proteins.

Early in the course of the present study, it became evident that a successful sterilizing technique would require high purification of β_1-metal-combining protein. Success of any given technique was judged by the relative lack of change of chemical and physical properties of the protein produced by the technique. It will, therefore, be necessary to describe both the properties and its method of purification.

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This work has been supported in the main by Grant H-2226 from the National Heart Institute, Public Health Service, and in part by grants from the Kresge Foundation, from the King Foundation, and by contributions from industry.

Received for publication Aug. 18, 1958.

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Physical and Chemical Properties of the Protein[11, 12].—Approximately 3 per cent of the total plasma proteins consist of β_1-metal-combining protein (transferrin) which comprises about 60 to 75 per cent of fraction IV-7,[13] the rest of the fraction being mainly albumin and α_2-globulin. This protein has been crystallized and it has been characterized as a β_1-globulin which differs widely in size, shape, solubility, and composition from the other β_1-globulins of human plasma. As it is capable of binding iron, copper, and zinc in vitro, it has been designated β_1-metal-combining protein. Its properties are summarized in Table I.

TABLE I. PROPERTIES OF β_1-METAL-COMBINING PROTEIN

Nitrogen content (Gm. N/Gm. protein)	0.147
Carbohydrate content (Gm. hexose/Gm. protein)	0.018
Extinction coefficient $E_{1\text{ cm.}}^{1\text{ per cent}}$ 280 mμ	11.2
Extinction coefficient $E_{1\text{ cm.}}^{1\text{ per cent}}$ 465 mμ	0.570
Iron binding capacity pH 7.4 (γFe/mg. protein)	1.25*
Copper binding capacity pH 8.6 (γCu/mg. protein)	1.42
Molecular weight	90,000†
Isoelectric point	
iron saturated	pH 5.45
iron free	pH 5.80
Sedimentation constant $s_{20,w}$	5.0
Electrophoretic mobility (veronal buffer pH 8.6, $\mu = 0.1$)	3.0×10^{-5} cm./volt/sec.

*Crystallized protein.
†Estimated from iron binding capacity.

As isolated from plasma in fraction IV-7, the protein is one-third saturated with iron. It has been demonstrated that the iron in the complex is in the ferric state and that the complex is ionic and not covalent. The maximum iron uptake corresponds to 2 atoms of iron per molecule of protein.

Purification of β_1-Metal-Combining Protein.—The solubility of β_1-metal-combining protein is a function of the degree to which it is saturated with iron. Careful investigation of the binding of iron by this protein and the dissociation of iron from the resulting complex has been made by Inman.[14, 15] Taking advantage of the solubility changes observed, he was able to isolate the pure protein in the iron-free form in good yield from fraction IV-7. We have followed Inman's technique which, in brief, is as follows.

METHODS

To a 20 per cent solution of fraction IV-7 in distilled water, adjusted to pH 7.4 with 0.1 N ammonium hydroxide, is added sodium bicarbonate to give a final concentration of 0.009 M. To this is added 1 Gm. $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ as a 2.5 per cent solution in 0.001 M acetic acid per 100 Gm. of fraction IV-7. This amounts to somewhat more than twice the stoichiometric amount of iron demanded by the metal-combining protein present. The mixture is then brought to pH 7.2 with 0.1 M ammonium hydroxide solution and allowed to stand at 0° to 2° C. for 12 hours.

The mixture is next dialyzed against running tap water or against repeated changes of distilled water for 40 hours at 0° to 2° C. It is then passed through a series of ion exchange resins in sequence in a single column at 0° to 2° C. Per 100 Gm. dry weight of fraction IV-7 the column contains from the base upward 20 ml. of IR-120 H^+,* a mixture of 60 ml. of IR-120 H^+ and 120 ml. of IRA-400 OH^-,* a mixture of 10 ml. of IR-120 NH_4^+ and 25 ml.

*Rohm & Haas Company, Philadelphia, Pa.

of IRA-400 CH_3COO^-. The low ionic strength obtained in the solution following this resin treatment is important, both in prevention of dissociation of Fe^{+++} from the metal-combining protein, and for increasing the precision of the ensuing separations.

The mixture is brought to 8.35 per cent protein concentration by dilution with cold distilled water. Per liter of solution, 392 ml. of 53.3 per cent ethanol is added at 0° C. to produce a final mixture of 6 per cent protein and 15 per cent ethanol at pH 5.2 to 5.4. The protein precipitate is discarded or reserved for reworking. The supernatant fluid is dried by lyophilization.

The lyophilized protein is dissolved in distilled water and rendered free from iron by the addition of EDTA (disodium salt of ethylenediaminetetraacetic acid). The conditions of the mixture are essentially 3.2 per cent protein, ionic strength 0.65 (acetate), pH 5.3 to 5.4, EDTA 5.0 mM., temperature 0° to 2° C. The mixture should stand 4 to 5 hours before precipitation of the purified β_1-metal-combining protein.

Precipitation of the metal-combining protein is achieved by adjustment of the mixture to pH 6.3 with 1 M $NaHCO_3$ solution, dilution to approximately 1 per cent protein concentration, and addition of sufficient 60 vol. per cent ethanol to produce an ethanol concentration of 37 per cent. The mixture should be kept close to its freezing point during and after the ethanol addition. The final temperature should be -7° to -9° C. The precipitate is collected by centrifugation at -7° C. and is triturated with 35 per cent ethanol to reduce the salt concentration. It is then dried by lyophilization. The final precipitate consists of 90 to 95 per cent β_1-metal-combining protein.

The above procedure is presented schematically in Table II.

Even with careful maintenance of low temperature throughout, there is the hazard that this relatively long isolation procedure may result in a pyrogenic product unsuitable for clinical study. Should this situation appear of paramount importance, the above procedure may be shortened by the less chemically elegant suggestions which follow.

The dialysis period prior to deionization may be eliminated. The layered resin column may be replaced with a column consisting of a mixture of equal parts (350 ml. per 100 Gm. of fraction IV-7) of IRC-120 H^+ and IR-45 OH^- resins. This mixture may be sterilized prior to use by autoclaving in the column. The solution to be deionized may be sterilized by Seitz filtration prior to passage through the column.

Analytic Procedures Used in Evaluating the Heated Protein.—1. Paper electrophoresis: All the preparations were examined in pH 8.6 barbital buffer of 0.075 ionic strength in a Durrum cell. 2. Tiselius electrophoresis: All preparations were run at 4° C. in pH 8.6 barbital buffer of 0.1 ionic strength. 3. Ultracentrifuge studies were performed in a Svedberg ultracentrifuge, the proteins being in 0.15 M sodium chloride solution. 4. Viscosity: Viscosities relative to distilled water were measured using 5 per cent solutions of protein in distilled water at 21° C. 5. Iron binding capacity (IBC): The formation of a colored complex with iron is a unique property of the β_1-metal-combining protein.[11] This property has been used as the primary measure of possible damage to the protein during heating.

Increasing amounts of a standard solution of ferrous ammonium sulfate in 0.001 M acetic acid are added to a 1 per cent solution of protein dissolved in tris(hydroxymethyl)aminomethane buffer of pH 7.4. The standard contains 800 μg of iron per milliliter. After one hour the optical density is read in a Beckmann spectrophotometer at 465 mμ against a protein-free blank (Fig. 1). The intersection of the two lines indicates the concentration of iron required to saturate the iron binding capacity of the protein and from this the percentage of the total protein present as iron binding protein can be calculated.

RESULTS

Heating of β_1-Metal-Combining Protein at 60° C. for 10 Hours.—The studies indicating the probable virucidal efficacy of the heating procedure used apply only to solutions of proteins. For that reason, all of the work reported has been performed with protein solutions.

Studies of the effect of the heating procedure were made with solutions of pure iron-free β_1-globulin and with iron-saturated β_1-globulin as fraction IV-7 and at various stages

TABLE II. SCHEMA FOR PURIFICATION OF β_1-METAL-COMBINING PROTEIN

Fraction IV-7

Protein	20 per cent
pH	7.45 ± 0.05
$NaHCO_3$	0.009 M
Fe^{+++}	0.0051 M
Temperature	0 to 2° C.
↓	
pH	7.2
Temperature	0 to 2° C. for 12 hr.
↓	
Dialyzed	40 hr.
Temperature	0 to 2° C.
↓	
Deionized through resin column 0 to 2° C. to specific resistance = 100,000 ohm-cm. (25° C.)	
↓	
pH	5.3 ± 0.1
Protein	6 per cent
Ethanol	15 per cent
Temperature	0° C.

Precipitate IV-7-1 contains some β_1-metal-combining protein which may be recovered

Supernatant IV-7-1

↓ Lyophilized

Protein	3 per cent
pH	5.35 ± 0.05
EDTA	5.0 mM
Temperature	0 to 2° C. for 5 hr.
↓	
Protein	1 per cent
pH	6.3
Ethanol	37 per cent
Temperature	−7° C.

Precipitate IV-7-2 90 to 95 per cent β_1-metal-combining protein

Supernatant IV-7-2 Discarded

of further purification. The data for pure iron-free protein are summarized in Fig. 2. For these studies, the protein was dissolved in distilled water and pH adjustments were made with 1 M $NaHCO_3$ or with pH 4 acetate buffer. Many of the heated samples changed visually in such a manner that the conditions under which they were heated could be eliminated from consideration. The study showed that for the pure iron-free protein concentrations greater than 1 per cent at the time of heating were undesirable, although higher concentrations of protein at low pH (between pH 4 and 5) might be tolerated. Analytic data for pure iron-free globulin after heating at pH 5.05 as a 5 per cent solution are given in Table III, Experiment 1. The sample, after heating, was homogeneous in the ultracentrifuge with no change of the sedimentation constant (Fig. 3). The Tiselius pattern showed possible slight change in mobility, however, and there was a loss of about 20 per cent of the iron binding capacity.

Johnson and Hink[16] had observed earlier that saturation of β_1-metal-binding globulin with iron increased its stability to heat. Studies were performed with iron-saturated globulin of varying degrees of purity and under various conditions of solution. These are summarized in Table III, Experiments 2, 3, 4, and 5. It is immediately evident that saturation of the protein with iron has much the same effect as the use of an efficient stabilizer has shown with other proteins. Iron binding capacity following heating remained unchanged without respect to protein concentration, state of purity, or pH (pH 5.9 to 7.15) at which the solutions were

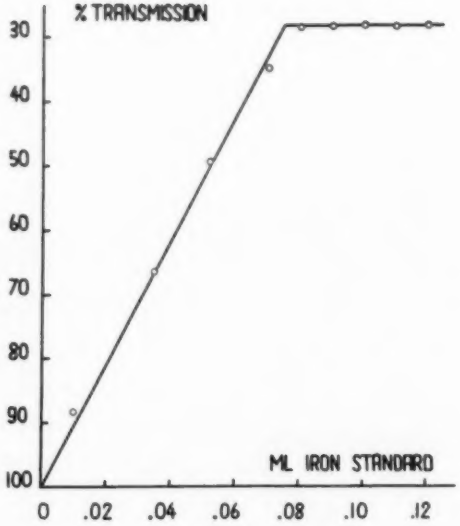

Fig. 1.

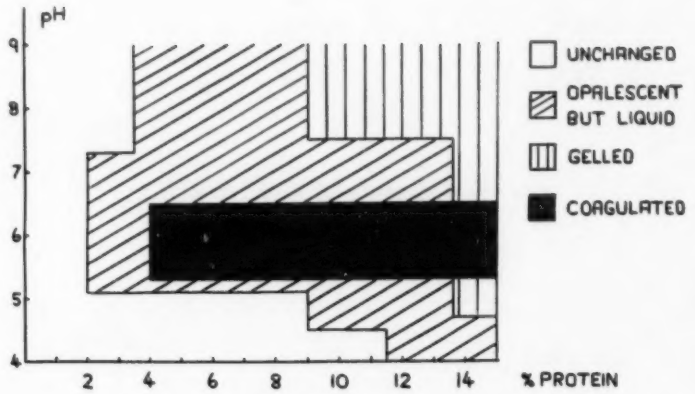

Fig. 2.

Fig. 1.—Measurement of iron binding capacity of 1 per cent solution of β_1-metal-combining protein. Per cent transmission at 465 mμ.
Fig. 2.—Changes observed during the heating of solutions of iron-free β_1-metal-combining protein for 10 hours at 60° C.

TABLE III. EFFECT OF HEATING FOR 10 HOURS AT 60° C. ON β_1-METAL-COMBINING PROTEIN

CONDITIONS OF HEATING					PROPERTIES OF THE ISOLATED β_1-METAL-COMBINING PROTEIN (IRON-FREE)								
								ELECTROPHORESIS % OF PROTEIN AS			MOBILITY ($\times 10^{-5}$ CM.^{2} SEC.$^{-1}$ VOLT^{-1})		
EXPERIMENT	FORM OF β_1-METAL-COMBINING PROTEIN	PROTEIN CONCENTRATION (GM. %)	pH	AMOUNT Fe^{+++}	IBC (γ Fe/MG. PROTEIN)	SEDIMENTATION CONSTANT	RELATIVE VISCOSITY	α_2-GLOBULIN	β_1-GLOBULIN	$\beta_2 + \gamma$-GLOBULIN	α_2-GLOBULIN	β_1-GLOBULIN	$\beta_2 + \gamma$-GLOBULIN
1	Pure	5.0	5.05	No iron	0.97	4.67			98.5	1.5		3.1	2.2
	Control unheated				1.20	4.84							
2	Fraction IV-7	18.0	7.08	Saturated	1.16								
	Control unheated				1.16								
3	Supernatant IV-7-1*	6.5	5.90	Saturated	1.18	4.86/7.01							
	Control unheated				1.18	4.82							
4	Fraction IV-7 low ionic strength	18.0	7.00	Saturated	1.19	4.67	1.446	5.8	92.2	2.0	4.2	3.0	2.1
5	Supernatant IV-7-1*	6.5	7.15	Saturated	1.16	4.51	1.411	8.0	90.0	2.0	4.7	3.5	2.6
	Supernatant IV-7-1*	18.0	7.00	Saturated	1.16	4.59	1.421	6.1	91.1	2.0	4.2	3.1	2.0
	Control unheated				1.20	4.59	1.398	2.7	95.2	2.1	3.8	3.1	2.4

*Lyophilized and reconstituted in H_2O to proper protein concentration and pH.

heated. Least change in viscosity was observed following heating at pH 7.15 as a 6.5 per cent solution. There were slight alterations in the percentage of protein migrating as β_1-globulin and as α_2-globulin in all heated samples. With the exception of globulin heated at pH 5.9, little change was noted in sedimentation constant following heating. The appearance of a second component in the ultracentrifuge was attributed to partial dissociation of iron at pH 5.9 during the heating process, permitting the iron-free globulin to aggregate.

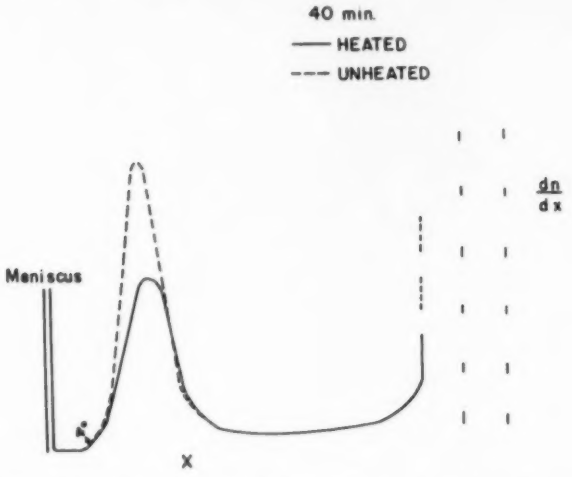

Fig. 3.—Comparison of tracings of ultracentrifuge patterns of heated and unheated iron-free β_1-metal-combining protein.

DISCUSSION

It is most desirable that any heat sterilizing technique for plasma proteins be applied to the final solution in the final bottle. In the case of β_1-metal-binding globulin this would necessitate application of the heating step to a neutral 15 per cent solution of the globulin in an isotonic menstruum. The globulin would be either iron-free or, at least, one-third saturated with iron as it appears to occur in vivo. From the above data, this most desirable technique is obviously impossible.

Heating of β_1-metal-binding globulin as fraction IV-7 would eliminate the necessity of the final purification which is probably not essential for clinical purposes. Table III, while not showing this the best procedure, does not indicate changes of property of the protein heated as fraction IV-7 that would necessarily preclude such a procedure. An attempt to prepare heated fraction IV-7 on a large scale indicated, however, that traces of other globulins denatured by the heating process were so serious a hindrance to sterilization of the final solution by filtration as to make the whole procedure impracticable.

Failing the above two possibilities, incorporation of heating as a step in purification would appear desirable. Various studies showed that heating for 10 hours at 60° C. after deionization but before precipitation of impurities gave

a satisfactory final product and, at the same time, increased the yield of β_1-metal-binding globulin, presumably by making the other globulins more readily separable by precipitation. It was necessary to adjust the pH of the mixture to 7.0 before the heating (the approximate conditions of Table III, Experiment 5) and back to pH 5.2 for the precipitation step. Iron was then removed from the protein in the course of subsequent purification. The iron-free β_1-metal-binding globulin obtained could be prepared readily as a sterile 15 per cent solution in 0.15 M NaCl for clinical investigation.

SUMMARY

It has been shown that the β_1-metal-combining protein may be heated at 60° C. for 10 hours for counteraction of possible contamination with hepatitis virus with essentially no alteration of the chemical and physical properties of the protein. This is best accomplished when the β_1-metal-combining protein is saturated with iron and during the process of its purification.

We wish to acknowledge gratefully the assistance of Dr. Efraim Toro-Goyco in performing the Tiselius electrophoretic analyses and of Mr. Charles Gordon in performing the ultracentrifugal analyses used in this report.

REFERENCES

1. Murray, R., Diefenbach, W. C. L., Geller, H., Leone, M. D., and Ratner, M. D.: The Problem of Reducing the Danger of Serum Hepatitis From Blood and Blood Products, New York State J. Med. **55**: 1145, 1955.
2. Murray, R.: *cited in* The Distribution of Viruses in Plasma Fractions (R. B. Pennell), Hepatitis Frontiers, Boston, 1957, Little, Brown & Company.
3. Scatchard, G., Strong, L. E., Hughes, W. L., Jr., Ashworth, J. N., and Sparrow, A. H.: The Properties of Solutions of Human Serum Albumin of Low Salt Content, J. Clin. Invest. **24**: 671, 1945.
4. Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G.: Influence of Non-Polar Ions on Thermal Stability of Serum Albumin, J. Clin. Invest. **23**: 54, 1944.
5. Gellis, S. S., Neefe, J. R., Stokes, J., Jr., Strong, L. E., Janeway, C. A., and Scatchard, G.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXVI. Inactivation of the Virus of Homologous Serum Hepatatis in Solutions of Normal Human Serum Albumin by Means of Heat, J. Clin. Invest. **27**: 239, 1948.
6. Surgenor, D. M., et al.: Protein-Metal Interactions in Protein Fractionation: Stable Plasma Protein Solution (SPPS) from Human Plasma. Separations with Zinc in Aqueous Media. (In preparation.)
7. Nitschmann, H., Kistler, P., Renfer, H. R., Hassig, A., and Joss, A.: A Heat Stable Human Plasma Protein Solution Obtained by Desalting (PPL), Vox Sang. **1**: (New Series): 183, 1956.
8. Mulford, D. J., Mealey, E. D., and Welton, L. D.: Preparation of a Stable Human Plasma Protein Solution, J. Clin. Invest. **34**: 983, 1955.
9. Hink, J. H., Jr., Hidalgo, J., Seeberg, V. P., and Johnson, F. F.: Preparation and Properties of a Heat-Treated Human Plasma Protein Fraction, Vox Sang. **2**: (New Series): 174, 1957.
10. Singher, Heron: Personal communication.
11. Surgenor, D. M., Koechlin, B. A., and Strong, L. E.: The Metal-Combining Globulin of Human Plasma, J. Clin. Invest. **28**: 73, 1949.
12. Koechlin, B. A.: Preparation and Properties of Serum and Plasma Proteins. XXVIII. The β_1-Metal-Combining Protein of Human Plasma, J. Am. Chem. Soc. **74**: 2649, 1952.
13. Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S., Jr., and Gibson, D. M.: Preparation and Properties of Serum and Plasma Proteins. XX. The Separation of Choline Esterase, Mucoprotein, and Metal-Combining Protein into Subfractions of Human Plasma, J. Am. Chem. Soc. **71**: 1223, 1949.
14. Inman, J. K.: Doctoral Thesis, Harvard Medical School, 1956.
15. Inman, J. K., and Oncley, J. L.: In preparation.
16. Johnson, F. F., and Hink, J. H., Jr.: Personal communication.

BLOOD COMPONENTS IN THE DOG: NORMAL VALUES

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SINCE dogs are used for investigations, the results of which may be applied to human beings, it is important to compare normal values for the various blood components. Although some of these data may be found in the literature,[1-3] population samples were often small and the various determinations were not done on the same animals. It is the purpose of this paper to present analytic data gathered over a period of several years and to compare these with values for human subjects.

METHODS

Our animals were obtained with no knowledge of previous history or dietary habits. They were, however, routinely dewormed and maintained on a standard diet of 7 to 10 ounces of a commercial dog food,* plus one ounce of raw horsemeat and not less than 1,500 ml. of water for a sufficient time to assure their general well-being.

Analytic methods were standard. Sodium and potassium were determined by flame photometry using a Beckman DU spectrophotometer with photomultiplier attachment and oxyhydrogen flame according to the method of Kingsley and Schaffert[4]; calcium and magnesium by an EDTA titration procedure developed in this laboratory[5]; chlorides by the method of Schales and Schales[6]; bicarbonate by the volumetric method of Van Slyke[7]; inorganic phosphate by the photometric method of Fiske and Subbarow[8]; proteins by the biuret method of Gornall, Bardawill, and David[9]; NPN by the procedure of Koch and McMeekin[10]; urea by the method of Karr[11]; creatinine by Peters' modification of the method of Folin and Wu[12]; thiosulfate[13] and T-1824[14] space by modifications developed in this laboratory; hematocrit by centrifuging 30 minutes in a Wintrobe tube at 3,000 r.p.m. at 2,000 g.[15]

RESULTS

Table I shows a comparison of the serum electrolytes of the dog with those of the human being. A wider range in most components is at once apparent. Significantly higher mean values for sodium and chloride ions were found in the dog and significantly lower potassium, bicarbonate, and protein values. With the exception of sodium, chloride, and bicarbonate, the mean values lie within the normal range of human values[16] found in this hospital. To establish these normal values, blood samples were obtained from 111 apparently healthy volunteers, consisting of employees and visitors of both sexes, ranging in age from 18 to 68 years.

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Presented at the 133rd National Meeting of the American Chemical Society, San Francisco, Calif., on April 15, 1958.
Received for publication Aug. 18, 1958.
*Gaines Dog Food was used.

TABLE I. SERUM ELECTROLYTES

SERUM ELECTROLYTES (MEQ./L.)	NUMBER OF SUBJECTS		MEAN VALUES		STANDARD DEVIATION		COMPLETE RANGE				95 PER CENT OF RANGE			
							HIGH		LOW		HIGH		LOW	
	DOG	HUMAN	DOG	HUMAN	DOG	HUMAN	DOG	HUMAN	DOG	HUMAN	DOG	HUMAN	DOG	HUMAN
Sodium	99	111	148.8	143.0	5.1	2.0	163.0	148.0	130.0	128.0	157.0	146.0	141.0	141.0
Potassium	99	111	3.8	4.4	0.5	0.3	5.1	5.3	2.3	3.6	4.7	5.0	2.6	3.9
Calcium	92	111	5.1	4.8	0.6	0.3	6.0	5.5	2.7	4.2	5.7	5.2	3.7	4.3
Magnesium	91	111	1.5	1.6	0.2	0.2	2.8	2.9	1.0	1.1	2.1	2.1	1.1	1.2
Chloride	93	111	111.0	106.0	6.0	3.0	120.0	108.0	87.0	95.0	119.0	106.5	100.0	97.6
Inorganic phosphate	73	111	2.3	2.2	0.7	0.3	4.1	3.0	0.8	1.3	3.8	2.7	1.1	1.7
Bicarbonate	23	111	20.6	26.1	2.7	1.7	25.9	30.8	15.7	22.6	25.4	28.5	17.1	23.4
Protein*	58	111	15.8	17.2	1.6	1.3	20.9	20.5	12.2	14.1	19.1	19.3	12.6	15.4

*These values were obtained by multiplying grams per cent by the Van Slyke Factor, 2.43.

TABLE II. NITROGENOUS CONSTITUENTS, PROTEIN COMPONENTS, AND BODY COMPARTMENTS

DATA	NUMBER OF SUBJECTS		MEAN VALUES		STANDARD DEVIATION		COMPLETE RANGE				95 PER CENT OF RANGE			
							HIGH		LOW		HIGH		LOW	
	DOG	HUMAN	DOG	HUMAN*	DOG	HUMAN	DOG	HUMAN*	DOG	HUMAN*	DOG	HUMAN	DOG	HUMAN
Nitrogenous Constituents (mg./100 ml.)														
NPN	100		27.5	25.0	6.5		52.4	29.0	15.3	22.0	42.4		16.7	
Urea	19		14.7	12.0	4.8		21.6	17.6	7.0	9.6	20.3		7.5	
Creatinine	59		1.2	1.1	0.47		2.7	1.3	0.3	1.0	2.6		0.4	
Protein Components (Gm./100 ml.)														
Total protein	58	111	6.5	7.1	0.64	0.53	8.2	8.6	5.0	5.8	7.9	7.9	5.2	6.3
Albumin	57	36	3.2	5.2	0.45	0.25	4.9	5.7	1.2	4.7	4.4	5.6	2.2	4.9
Globulin	57	36	3.3	2.0	0.86	0.27	5.6	2.5	1.7	1.3	5.5	2.4	2.1	1.4
Body Compartments* (ml./kg.)														
Hematocrit	91		45.4	44.5	5.0		59.0	54.0	34.0	37.0	56.0		35.0	
Plasma volume*	29	90	48.2	44.8	8.0		66.0	55.0	32.0	34.0	64.0		37.0	
Extracellular fluid*	23	19	228.0	235.0	30.0		310.0	260.0	172.0	210.0	276.0		182.0	

*Normal Values in Clinical Medicine, Sunderman and Boerner.

The remaining constituents presented in Table II show an even wider range for normal dogs than for normal human subjects. Since the nitrogenous constituents, as reported by Sunderman and Boerner,[2] were gleaned from various sources, numbers and standard deviations were not always available. The mean values found for the dog, however, lie well within the normal human range.

In considering the protein components, it is again apparent that not only are the ranges of normal values of serum total protein, albumin, and globulin wider for the dog than those found for human beings, but also that the means differ considerably. The mean serum total protein of the dog is only about 90 per cent of that of the human; the albumin is about 60 per cent of that of the human; and the globulin (found by difference) more than 150 per cent that of the human subject.

Normal values of the body compartments also show a somewhat wider range for the dog than those for human beings. However, their mean values show a very close agreement.

Since many of the blood specimens were obtained from dogs which had been fasted from both food and water for 24 hours or longer, it was thought advisable to determine if this might have had any effect on the results. For this purpose a group of 4 dogs was bled, fasted for 24 hours, and bled again. This procedure was repeated the following week. Hematocrits, serum sodium, potassium, calcium, magnesium, phosphate, chloride, nonprotein nitrogen, creatinine, total protein, and albumin were determined on each of the 16 specimens. The results, upon being subjected to statistical analysis, showed no significant difference between the means of the fasting and nonfasting specimens.

DISCUSSION

Possible explanations for the differences between normal dog and normal human serum include the presence of parasites, inadequate diet in the animals' previous environment, and inherent differences in the species. The first of these might be eliminated by efficient medication and the second by maintenance on a proper diet for a long period of time. Low A/G ratios remained constant, however, in animals kept for some months.

The relationship between the hematocrit and the A/G ratio is shown in Fig. 1. This graph has a standard error of estimation of 4.8 and a coefficient of correlation of 0.60. This is significant at the 1 per cent level of probability. The relationship suggests that a hematocrit determination might be used as a simple screening procedure for hypoproteinemia. Although protein fractionation by saturated sodium sulfate may be imperfect, Deutsch and Goodloe[17] found that the electrophoretic pattern for dog plasma showed a close resemblance in separation and mobility of components to that of human beings.

Since plasma volume may be estimated from weight for the human as reported by Inkley, Brooks, and Krieger,[18] a similar equation was derived for the dog, as shown in Fig. 2. The coefficient of correlation of this curve is

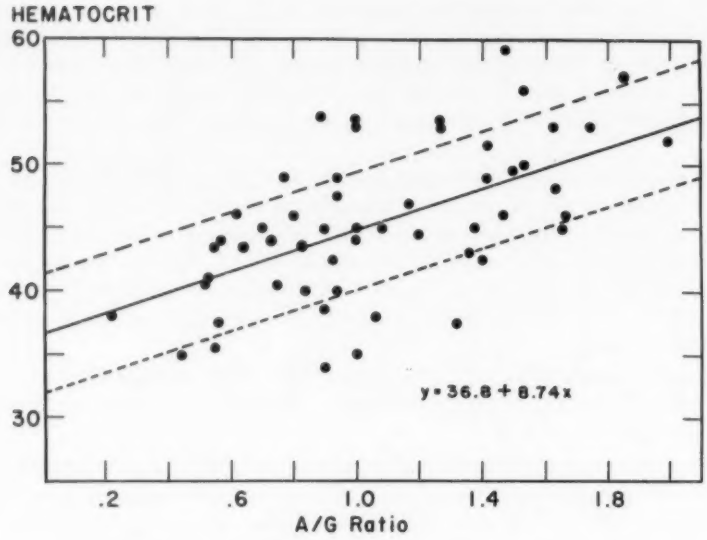

Fig. 1.—The relationship between hematocrit and A/G ratio.

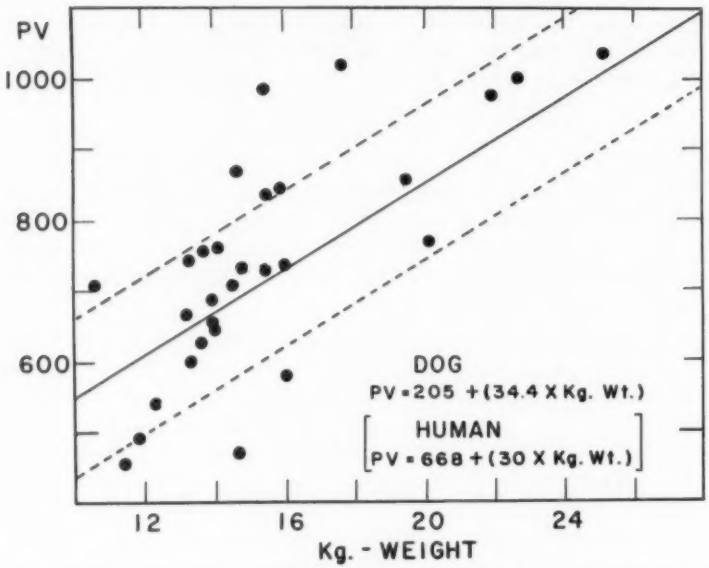

Fig. 2.—The relationship between plasma volume and body weight. Human data is that of Inkley and associates.[18]

0.73 which is significant at the 1 per cent level of probability. However, the standard error of this estimation is 111 and plasma volume can be estimated from weight not more accurately than ±16 per cent. The mean plasma volume was 48 ml. per kilogram.

In a similar manner, extracellular fluid volume or thiosulfate space, was related to plasma volume (Fig. 3). The standard error of this estimate is 120 and the coefficient of correlation is 0.75 which is significant at the 1 per cent level of probability. If it is desired to estimate extracellular fluid volume from weight, the value of plasma volume expressed in terms of weight may be substituted in this equation giving: $V_{ec} = (294 \times \text{wt. in Kg.}) - 838$. Again the error may be as high as ±25 per cent.

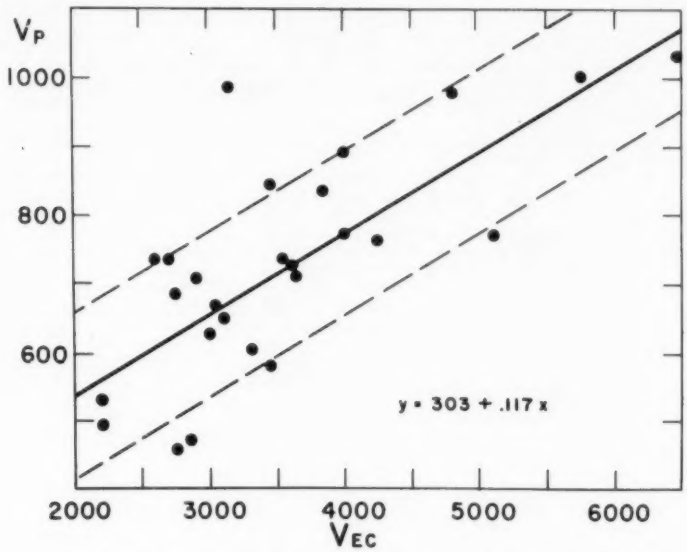

Fig. 3.—The relationship between plasma volume and extracellular fluid.

SUMMARY

1. There are statistically significant differences between some of the serum electrolytes of dogs and human subjects, but with the exception of sodium and chloride and the protein components, the mean values for the blood components of the dog lie well within the normal range for corresponding components in the human.

2. There is a correlation between the hematocrit and the A/G ratio, between plasma volume and body weight, and between plasma volume and extracellular fluid volume.

3. Plasma volume and extracellular fluid volume may be roughly estimated from body weight.

REFERENCES

1. Albritton, E. C.: Standard Values in Blood, Philadelphia, 1952, W. B. Saunders Company.
2. Sunderman, F. W., and Boerner, F.: Normal Values in Clinical Medicine, Philadelphia, 1950, W. B. Saunders Company.
3. Baer, J. E., Peck, H. M., and McKinney, S. E.: Some Normal Laboratory Values in the Dog, Proc. Soc. Exper. Biol. & Med. **95**: 80-84, 1957.
4. Kingsley, S. R., and Schaffert, R. R.: Direct Microdetermination of Sodium, Potassium, and Calcium, Anal. Chem. **25**: 1738-1741, 1953.
5. Carr, M. H., and Frank, H. A.: An Improved Method for Determination of Calcium and Magnesium in Biologic Fluids by EDTA Titration, Am. J. Clin. Path. **26**: 1157-1168, 1956.
6. Schales, O., and Schales, S. S.: A Simple and Accurate Method for the Determination of Chloride in Biological Fluids, J. Biol. Chem. **140**: 879-884, 1941.
7. Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry, ed. 13, New York, 1954, Blakiston Company (Division of Doubleday & Company, Inc.), pp. 693-700.
8. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375-400, 1925.
9. Gornall, A. B., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751-766, 1949.
10. Koch, F. C., and McMeekin, T. L.: A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler Folin Reagent for Ammonia, J. Am. Chem. Soc. **46**: 2066-2069, 1924.
11. Karr, W. G.: A Method for the Determination of Blood Urea Nitrogen, J. Lab. & Clin. Med. **9**: 329-333, 1923-1924.
12. Peters, J. H.: The Determination of Creatinine and Creatine in Blood and Urine With the Photoelectric Colorimeter, J. Biol. Chem. **146**: 179-186, 1942.
13. Frank, H. A., and Carr, M. H.: A Simplified Method for Estimation of the Extracellular Fluid Space, J. Lab. & Clin. Med. **45**: 973-976, 1955.
14. Frank, H. A., and Carr, M. H.: A Simplified Dye Method for Estimating Plasma Volume, J. Lab. & Clin. Med. **45**: 977-980, 1955.
15. Vasquez, O. N., Newerly, K., Yalow, R. S., and Berson, S. A.: Determination of Trapped Plasma in the Centrifuged Erythrocyte Volume of Normal Human Blood with Radioiodinated (I^{131}) Human Serum Albumin and Radiosodium (Na^{24}), J. Lab. & Clin. Med. **39**: 595-604, 1952.
16. Frank, H. A., and Carr, M. H.: "Normal" Serum Electrolytes With a Note on Seasonal and Menstrual Variation, J. Lab. & Clin. Med. **49**: 246-252, 1957.
17. Deutsch, H. F., and Goodloe, M. B.: An Electrophoretic Survey of Various Animal Plasmas, J. Biol. Chem. **161**: 1-20, 1945.
18. Inkley, S. R., Brooks, L., and Krieger, H.: A Study of Methods for the Prediction of Plasma Volume, J. Lab. & Clin. Med. **45**: 841-850, 1955.

CLEARING OF SMEARS MADE FROM WOUND EXUDATES

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DURING the course of a study of experiental wounds in the goat,[1] we have examined more than 2,000 smears of wound exudates. Early in the course of this study it became apparent that the data obtained from examination of simple stained smears were of limited value. The exudates contained so much debris that it was difficult to identify, and virtually impossible to estimate the concentration of, the organisms present (Figs. 1 to 3 and 9, *A*). The debris can be attenuated by dilution, but even so it is difficult to identify accurately gram-negative rods and nonchained cocci when there is any significant amount of background material. And, unfortunately, the organisms are diluted as easily as is the debris.

One of us (J. H. T.) had worked with the late Dr. Stanley H. Durlacher and was familiar with Durlacher's[2] application of the Spalteholz[3] technique to the clearing of coronary arteries. After a series of trials we have succeeded in adapting to bacteriology this technique of anatomy and gross pathology.

METHODS

1. Air dry smear at room temperature.
2. Fix dried smear for 1 minute in 95 per cent ethanol.
3. Rinse fixed smear in chloroform for 5 to 10 seconds.
4. Soak for 5 to 15 minutes in tap water; the time varies with amount of blood or other proteinaceous material in smear.
5. Air dry smear at room temperature.
6. Use Gram's stain on the dried smear and air dry again.
7. Clear smear in mixture of equal parts of methyl salicylate and methyl benzoate.

The time required for optimum clearing will vary from 5 minutes to 6 hours or more, depending on the density of the smear. We normally use a 20 minute clearing period, which seldom results in overdecolorization (Figs. 2 and 6, 3 and 7). If the smear is unusually dense, it may be returned to the mixture for further clearing (Figs. 1 and 5). If the smear is overdecolorized it can be restained, and cleared again.

RESULTS AND DISCUSSION

Typical results are illustrated in the figures.

We have found the method to be consistently reproducible over the wide range of wound exudates which we have been studying. Our experience with exudates from other sources is limited, but it is sufficient to indicate that the

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Received for publication Aug. 20, 1958.

Fig. 1.—Uncleared smear, 16 hours after wounding; animal treated with penicillin.
Fig. 2.—Uncleared smear, 6 hours after wounding; no antibiotic.
Fig. 3.—Uncleared smear, 9 hours after wounding; animal treated with penicillin.
Fig. 4.—Smear 66 hours after wounding, decolorized with H_2O_2 and acetone-alcohol; most of the field is in focus indicating thinness of smeared material; many organisms are decolorized.

Fig. 5.—Replicate of smear shown in Fig. 1, cleared for 6 hours in methyl salicylate and methyl benzoate.
Fig. 6.—Replicate of smear shown in Fig. 2, cleared for 20 minutes in methyl salicylate and methyl benzoate.
Fig. 7.—Replicate of smear shown in Fig. 3, cleared for 20 minutes in methyl salicylate and methyl benzoate; numerous gram-negative bacilli are visible.
Fig. 8.—Replicate of smear shown in Fig. 4, cleared for 12 hours in methyl salicylate and methyl benzoate; smear is much thicker, and fewer organisms are decolorized.

A. *B.* *C.*

Fig. 9.—Smear of wound exudate, 10^{-1} dilution. All photographs are of the same microscopic field. Uncleared smear (*A*) is unreadable due to great amount of debris. Cleared smear (*B*) is adequate for quantitative estimation and morphologic study. At a higher level of focus (*C*) it is evident that the dark mass in the cleared smear is not uncleared material, but consists of piled-up organisms, principally gram-positive rods. (Exposure times [color film]: *A*, 30 sec.; *B* and *C*, 18 sec.)

technique may be of wide usefulness in both the experimental and the clinical laboratory. It is of particular value in the preparation of smears for quantitative study.[4]

Even when the smear is not grossly dense, on uncleared smears we have often completely missed gram-negative organisms occurring in concentrations as high as 1,000 per oil immersion field.

We have tried other methods of clearing smears of wound exudates, and we have found none which produces results of comparable quality. Simple immersion of the gram-stained slide in chloroform for 5 to 10 minutes will produce a marked degree of clearing, but the final result is a somewhat cloudy, gray slide with poor gram specificity. Decolorization with H_2O_2 and acetone-alcohol causes some loss of the smeared material, and decolorizes organisms as well as debris (Figs. 4 and 8).

SUMMARY

By a simple adaptation of the Spalteholz technique, bacteriologic smears which are unreadable due to debris can be cleared to permit adequate study (Fig. 9).

We are indebted to Dr. Stanley C. Benbrook, Dr. Ward R. Richter, and Dr. Frank B. Johnson for assistance and encouragement.

REFERENCES

1. Lindsey, D., Wise, H. M., Jr., Knecht, A. T., and Noyes, H. E.: The Role of Clostridia in Mortality Following an Experimental Wound in the Goat. I. Quantitative Bacteriology. (Unpublished data.)
2. Durlacher, S. H., Fisk, A. J., and Fisher, R. S.: Lesions of the Coronary Arteries, An Improved Technic for Their Demonstration, Lab. Invest. **2**: 261, 1953.
3. Spalteholz, W.: Über das Durchsichtigmachen von menschlichen und tierischen Präparaten und sein theoretischen Bedingungen, ed 2, Leipzig, 1914, Hirzel.
4. Lindsey, D.: Quantitative Bacteriological Study of Tissue, Fluids, and Exudates: Some Simplified Methods, J. Lab. & Clin. Med. **53**: 299, 1959.

A RAPID METHOD FOR S^{35} RADIOASSAY AND GRAVIMETRIC SULFUR DETERMINATION IN BIOLOGIC MATERIAL

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LOS ANGELES, CALIF.

SULFUR can be combusted to sulfate by either a dry or a wet ashing technique. In metabolic studies where body fluids and tissue extracts in fairly large volume may be used, the wet ashing procedure is more convenient and does not require the use of specialized equipment. The combustion fluids used by previous workers for wet ashing were perchloric-nitric acid[1, 2] and fuming nitric acid-hydrogen peroxide.[3] The procedure employing perchloric-nitric acid appears to be the simplest and is the most widely used; however, there seems to have been no critical study of the procedure. To adapt the method for routine simultaneous analysis of radioactive sulfur in plasma and tissue protein samples, conditions of combustion were investigated and the procedure greatly simplified.

In the majority of published methods for determining the specific activity of S^{35}, sulfate has been collected either as the barium or benzidine salt. With conventional filter paper, handling of small precipitates of benzidine sulfate is somewhat easier than barium sulfate. However, perchlorate interferes with benzidine precipitation and has to be completely destroyed. Use of glass filter paper described here obviates the difficulties encountered in the filtration of barium sulfate. With the present procedure, a technician in a working day can combust and mount for radioassay barium sulfate from approximately 30 samples.

MATERIALS AND METHODS

Reagents.—

Modified combustion mixture[4]: Two volumes of concentrated nitric acid to 1 volume of 70 per cent perchloric acid, containing 10 mg. of copper nitrate, $Cu(NO_3)_2 \cdot 3H_2O$, per milliliter. (Use finely ground copper nitrate.)

Ammonium vanadate: 1 per cent in 20 per cent nitric acid.

Barium chloride solution: 10 per cent.

Hydrochloric acid: 0.5 N.

Ethyl alcohol 95 per cent and acetone.

Glass filter paper: Hurlbut 934 AH* or Schleicher & Schuell No. 23.†

Sintered glass filter funnels for the collection of the precipitate have been described elsewhere.[4]‡

From the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif. This work has been supported by a grant from the United States Public Health Service.
Received for publication Aug. 26, 1958.

*Distributed by H. Reeve Angel & Company and Fisher Scientific Company.

†Available from Schleicher & Schuell, Keene, N. H.

‡Available from California Glassblowing, 35 Raymond Street, Pasadena, Calif.

Hot plate with a variable temperature control.

Combustion.—Samples containing up to 0.5 Gm. of organic matter and up to 5 mg. of sulfur are transferred into 50 ml. Erlenmeyer flasks. For nonprotein sulfur determination, plasma or tissue extracts are deproteinized with 5 per cent perchloric acid. If the samples contain less than 0.5 mg. of sulfur, carrier sodium sulfate solution is added.

About 5 ml. of combustion fluid per 2 ml. of plasma or 100 mg. of organic matter and 2 drops of vanadate solution are added and the flasks heated on a hot plate located in a well-ventilated hood. The rate of heating is adjusted so that within 30 to 60 minutes the solutions are evaporated down to 1 to 2 ml.*

The temperature of the hot plate is turned down so that most of the dense perchloric acid fumes condense in the neck of the flask and the flasks are covered with glass marbles. The samples are digested for 2 or more hours. The marbles are then removed and the hot-plate temperature increased to evaporate most of the perchloric acid (about half an hour). A wet greenish-yellow to orange residue is obtained. The flasks are removed from the hot plate, about 10 ml. of water is added (see below), and it is returned to the hot plate. The residue should dissolve readily. One milliliter of barium chloride is added to the hot solution to precipitate the sulfate.

If the flasks were overheated and all the perchloric acid driven off, the residue may turn yellow, red, or black and does not dissolve. Addition of a few drops of perchloric acid and heating to obtain a clear solution is then required.

Filtration.—Preweighed glass filter paper is put on a sinter, wetted, suction applied, and the funnel clamped in place. The precipitate is poured on to the filter, washed successively with 0.5 N hydrochloric acid, water, alcohol, and acetone, and finally dried (with the suction on) with an infrared lamp. The filtration and drying require 2 to 3 minutes each, and, using two filters, 20 to 25 precipitates can be mounted in one hour.

The papers are reweighed to determine the weight of the precipitates, and the $sulfur^{35}$ activity is assayed.

DISCUSSION

Glass Filter Paper.—These papers have recently become commercially available and have been used to filter proteins for radioassay.[2] They combine a high retentivity with a high filtration speed and are not hygroscopic. For the handling of radioactive precipitates, they have the advantage of remaining perfectly flat upon drying. Thus, heavy deposits of barium carbonate and sulfate can be obtained without any cracking or fissuring of the precipitates.

Some glass filter papers are rather soft and tear readily. In this laboratory, the Hurlbut paper and the Schleicher & Schuell No. 23 paper have been used. The former paper has been quite variable in weight and quality and some batches have not been satisfactory.

The No. 23 Schleicher & Schuell paper is a smooth, thin, dense paper of uniform quality. In spite of its thinness (16 to 18 mg. per 2.4 cm. paper), it is very retentive and has good wet strength. It apparently contains a filler that is leached out during the filtration procedure, losing about 1 mg. in weight. Hence, blank papers should be included in each run, or the papers prewashed. Such prewashed papers do not change subsequently in weight.

Glass filter paper is commercially available in sizes of 2.1, 2.4, and 3.7 cm. Most commercial Geiger tubes accommodate a 1 or a 1¼ inch planchet, and the 2.4 cm. papers can be conveniently assayed in these planchets.

*With solutions containing organic solvents or very finely pulverized material, the initial reaction may be violent and these samples are predigested with 5 to 10 ml. of 50 per cent nitric acid prior to addition of the combustion fluid.

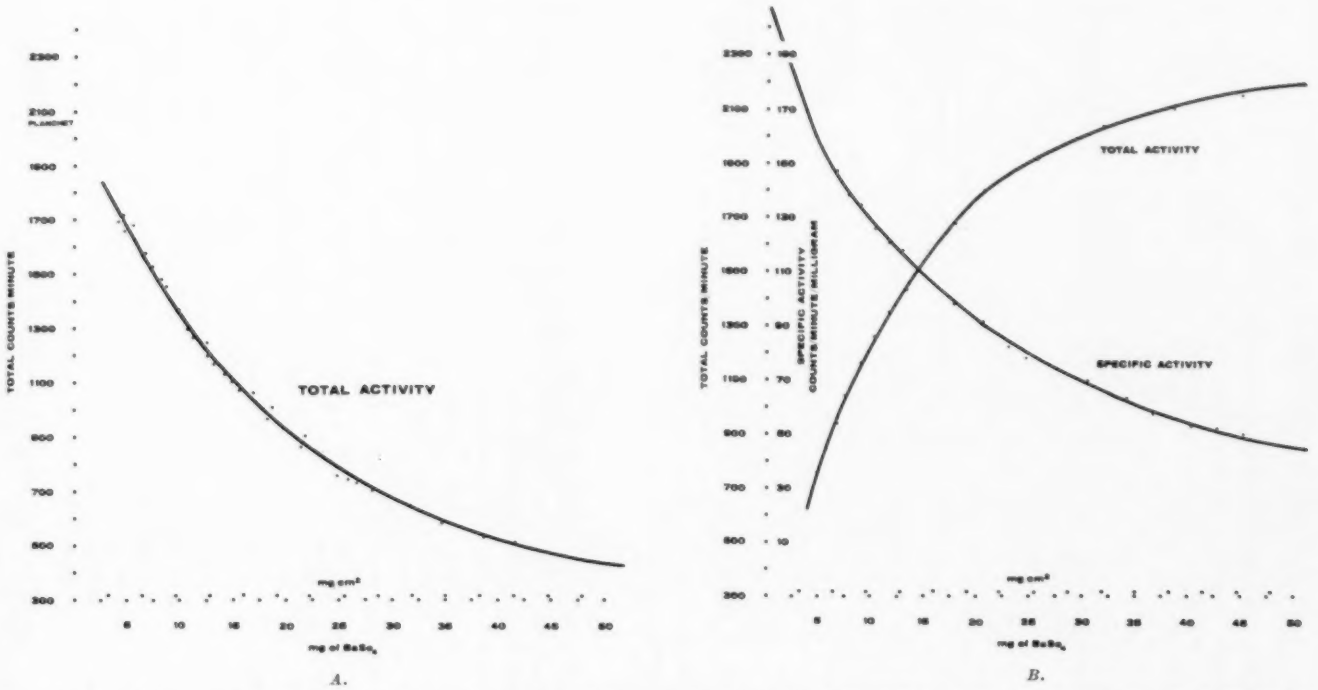

Fig. 1.—Self-absorption of $BaS^{35}O_4$. *A*, Unlabeled sulfate added to a constant amount of S^{35}-labeled sulfate. *B*, Precipitation of an S^{35}-labeled sodium sulfate solution. Total and specific activities of $BaSO_4$. Diameter of precipitate 2.0 cm. (No. 23 Schleicher & Schuell glass filter paper, 2.4 cm.). Activity assayed with Nuclear-Chicago gas flow counter with Micromil window.

Precipitation of Barium Sulfate.—In standard analytic procedures, it is recommended to precipitate barium sulfate slowly from hot solutions. However, with glass filter paper, the conditions for precipitation are not critical. With 50 μM of sulfate, theoretical yields were obtained whether the barium chloride was added fast or slow and regardless of the temperature. Provided the solutions were at least 0.1 N in perchloric acid and contained excess barium, results were not affected by thirty to fifty fold variations of acid and barium concentrations.

The only factor requiring attention is the concentration of sulfate. When its concentration is below 0.005 M, the recovery of sulfur^{35} activity is low. Thus, the amount of water added after digestion should be such as to yield more than 1 to 2 mg. of $BaSO_4$ per milliliter of final volume.

To test the reproducibility of the barium sulfate collection, a constant amount of carrier-free sulfate^{35} was added to increasing amounts of sodium sulfate and the precipitate weighed and counted. In Fig. 1, observed counts versus weight of barium sulfate are plotted. The experimental values fall on a smooth curve which approximates a straight line up to 16 mg. of $BaSO_4$. The deviations of observed values from the curve rarely exceed the error due to counting alone (3 per cent). The extrapolated value to 0 mass is about 6 per cent lower than the count obtained by directly evaporating the carrier-free sulfate^{35} on an aluminum planchet. This difference can be accounted for due to the higher backscattering of aluminum as compared to paper.

Conditions of Combustion.—The recovery of amino acid and protein sulfur was found to depend upon: (1) nature of compounds; (2) ratio of combustion fluid to organic material; and (3) time of digestion. Methionine sulfur is more slowly oxidized to sulfate than cysteine sulfur. Under the above conditions, the oxidation of methionine sulfur to sulfate was 85 to 90 per cent complete after an hour's digestion, and complete within 2 hours. Ammonium vanadate has been recommended as a catalyst to increase the rate of perchloric acid combustion.[5] We found it to have little effect on the rate of combustion, but the combustion proceeds more smoothly in its presence.

TABLE I. DETERMINATION OF SULFUR IN METHIONINE

GLUCOSE (MG.)	COMBUSTION FLUID (ML.)	TIME OF DIGESTION (HOURS)	$BaSO_4$ YIELD (MG.)	PER CENT OF THEORETICAL RECOVERY
0	5	2	23.3	100
0	5	20	23.1	99
100	5	2	23.3	100
100	5	20	23.1	99
500	20	2	23.6	101
500	20	20	23.0	99

Sample combusted 14.92 mg. of L-methionine (certified grade, 40.08 per cent; purchased from California Biochemical Foundation). Each value is an average of a triplicate determination.

In Table I, results with methionine combustion in the presence of varying amounts of organic matter (added glucose) are given. The recoveries of

sulfur were complete in all cases whether the samples were combusted for 2 hours or overnight. In Table II, replicate combustions of labeled kidney tissue are presented.

TABLE II. COMBUSTION OF S^{35} PROTEIN-LABELED KIDNEY TISSUE (SULFATE CONTENT AND SPECIFIC ACTIVITY)

WEIGHT OF TISSUE (MG.)	VOLUME OF COMBUSTION FLUID (ML.)	TIME OF DIGESTION (HOURS)	YIELD OF $BaSO_4$ (MG.)	SULFUR CONTENT OF KIDNEY (PER CENT)	OBSERVED SPECIFIC ACTIVITY (C./MG./MIN.)*	STANDARD SPECIFIC ACTIVITY (C./MG./MIN.)*
50	5	2	3.4	0.94	315	216
100	5	2	7.1	0.96	289	237
200	10	2	14.7	1.00	204	227
300	15	2	22.4	1.02	157	228
400	20	2	30.6	1.03	130	236
Average		2		0.99		229
50	5	20	3.4	0.94	362	248
100	5	20	6.9	0.95	287	234
200	10	20	14.5	0.99	204	227
300	15	20	22.0	1.01	157	225
400	20	20	29.3	1.02	126	225
Average				0.98		232

Desiccated ground kidney tissue from rabbit injected with S^{35}-labeled plasma. $BaSO_4$ mounted on 2.4 cm. glass filter paper. S^{35} assayed with Nuclear-Chicago gas flow counter with Micromil window. Standard specific activity (at 12 mg. $BaSO_4$, 3.82 mg./cm.^{2}) obtained by means of Table III.

*Counts per milligram per minute.

When trace amounts of sulfur^{35} were ashed and carrier sulfate added before precipitation, lower counts were obtained than when carrier sulfate was added prior to combustion. It appears that small amounts (of the order of a few micrograms) of sulfate can be lost during prolonged combustion, and, accordingly, trace amounts of sulfur^{35} should be combusted in the presence of carrier.

Self-absorption of Barium Sulfate S^{35}.—To compare the activity of sulfate S^{35} samples, it is necessary to correct for self-absorption and convert all counts to a common base (such as counts at zero mass, infinite thickness, or any conveniently chosen mass).

Fig. 1, *A* and *B*, illustrates the self-absorption characteristics of the weak beta activity in barium sulfate. In *A*, increasing amounts of unlabeled sulfate have been added to a constant amount of labeled sulfate. In *B*, the change in specific activity with increasing weight of $BaSO_4$ is shown. The two curves are of similar shape, both decline linearly until about 16 mg. weight (5 mg./cm.^{2}) and then both flatten out. "Infinite thickness" is attained at about 20 mg. per cm.^{2}.

From Fig. 1, *A* or *B*, correction tables such as Table III can be constructed to convert the observed count at any one mass to a desired "standard" count. We have adopted the total count (or the specific activity) obtained with a mass of 12 mg. of barium sulfate (3.82 mg. per cm.^{2}) as our standard. The observed activity at any one weight is multiplied by the corresponding factor from Table III to obtain the standard activity at 12 mg.

In the last column of Table II, we, in this way, have converted the observed specific activities of barium sulfate to our standard values. It can be seen that, although the amounts of tissue combusted and the $BaSO_4$ yield varied eightfold, the corrected specific activity of the $BaSO_4$ was quite uniform, the standard error not exceeding the counting error. When the range from 7 to 30 mg. is considered, the agreement is even closer, the deviation not exceeding 3 per cent.

TABLE III. CONVERSION TABLE FOR SPECIFIC ACTIVITIES

MILLIGRAMS	0	0.2	0.4	0.6	0.8
0	0.605*				
4	0.71	0.715	0.725	0.735	0.74
5	0.745	0.75	0.76	0.77	0.775
6	0.78	0.79	0.795	0.805	0.815
7	0.82	0.825	0.83	0.84	0.85
8	0.855	0.86	0.865	0.87	0.88
9	0.89	0.90	0.91	0.915	0.20
10	0.93	0.94	0.945	0.95	0.96
11	0.965	0.97	0.975	0.98	0.99
12	1.00	1.005	1.01	1.02	1.03
13	1.04	1.045	1.05	1.06	1.07
14	1.08	1.09	1.10	1.105	1.11
15	1.12	1.125	1.13	1.14	1.15
16	1.16	1.165	1.17	1.18	1.19
17	1.20	1.21	1.22	1.23	1.24
18	1.245	1.25	1.26	1.27	1.28
19	1.29	1.295	1.30	1.31	1.325
20	1.335	1.34	1.35	1.36	1.37
21	1.38	1.39	1.395	1.40	1.405
22	1.425	1.430	1.44	1.45	1.46
23	1.47	1.48	1.49	1.50	1.51
24	1.52	1.53	1.54	1.55	1.56
25	1.57				

Multiply the specific activity (or total count) obtained at given weight with factor from table to obtain specific activity or count with 12 mg. $BaSO_4$ (3.82 mg./cm.^{2}). Data obtained with a precipitate area of 2 cm. diameter.

*Obtained by extrapolation.

Size of Sample.—With samples of less than 3 mg. of barium sulfate, results were erratic, so this amount is minimal. The precision of the method is determined by the weighing error which, in the 3 to 5 mg. range is 5 to 10 per cent. Above 10 mg. the weighing error is not significant as compared with the statistical counting error (3 to 5 per cent).

As can be seen from Fig. 1, there is little gain in counting efficiency if the weight of the $BaSO_4$ is increased above 20 mg. If the extrapolated specific activity at 0 mass is taken as 100, the specific activity is 73 per cent at 10 mg., 59 per cent at 15 mg., and 37 per cent at 30 mg. Doubling sample size from 15 to 30 mg. increases the total count rate by only 25 per cent. Thus, the use of samples yielding more than 20 mg. offers no advantages.

S^{35} Assay of Samples Low in Sulfate.—The sulfur content of most biologic tissues is low and rarely exceeds a few per cent. Often there is not enough material to obtain sufficient precipitate and, in such cases, the addition of carrier sulfate is required.

Such samples are often assayed by directly counting the material.[6] However, the preparation of these samples, and especially the even distribution of material on disks or planchets, is difficult, and such methods are less reliable than those using barium sulfate. Indeed, when S^{35} incorporation into heterogenous fractions is studied, the combustion of material to sulfate is essential for accurate work. Enough carrier sulfate is added to yield 10 to 15 mg. precipitate. The recovery of the sulfate is quantitative, and the activity of the samples can be readily obtained.

SUMMARY

A wet-ashing procedure for the combustion of biologic material to sulfate and the assay of S^{35} activity is described. The conditions for the perchloric-nitric acid combustion of tissues have been investigated, and the procedure has been simplified to permit the routine simultaneous determination of sulfur and the assay of sulfur^{35} activity in a large number of samples. Sulfate is precipitated as barium sulfate and plated on glass filter paper. Use of glass filter paper eliminates the difficulties commonly encountered in the handling of such precipitates for both gravimetric sulfate determination and assay of radioactive sulfate.

REFERENCES

1. Lematte, L., Bionot, G., and Kahane, E.: La disage du soufre total des tissus et des aliments, Compt. rend. Soc. de biol. **96**: 1211, 1923.
2. Pirie, N. W.: Studies in Sulphur Metabolism of Dog; Metabolism of Methionine and Related Sulphides, Biochem. J. **26**: 2041, 1932.
3. Dohlman, C. H.: A Method for Assaying Small Amounts of S^{35} in Tissues, Ark. Kem. **11**: 225, 1957.
4. Katz, J., Sellers, A. L., and Marmorston, J.: A Rapid Method for Preparation of Plasma Proteins for Radioassay, J. Lab. & Clin. Med. **50**: 489, 1957.
5. Smith, G. F.: The Wet Ashing of Organic Material Employing Hot Concentrated Perchloric Acid, Anal. chim. acta **8**: 397, 1951.
6. Poddar, R. K.: Quantitative Measurement of S^{35} in Biological Samples, Nucleonics **15**: 82, 1957.

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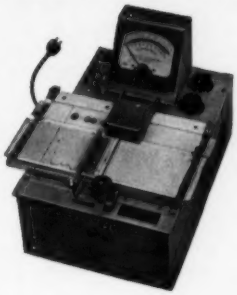

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University Hospital
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1313 Ann Street
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PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD.
ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

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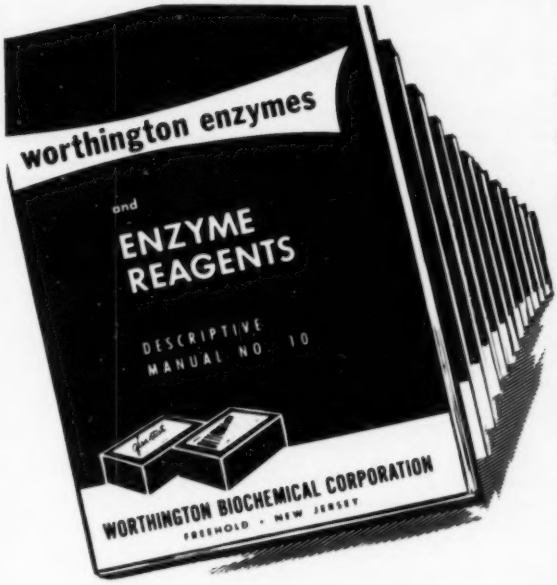

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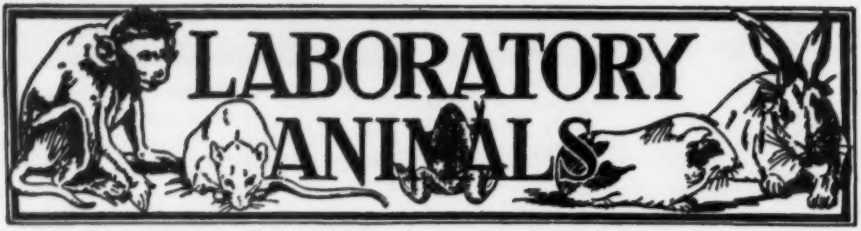

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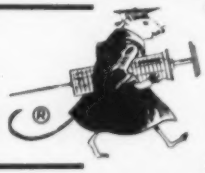

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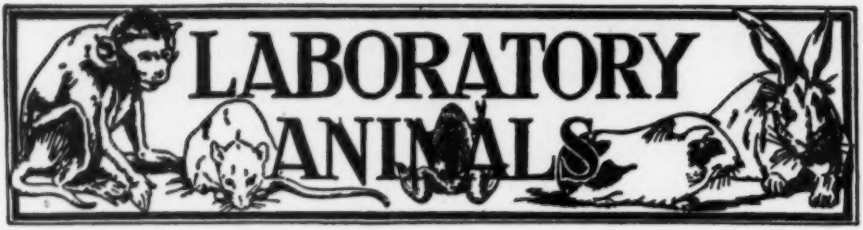

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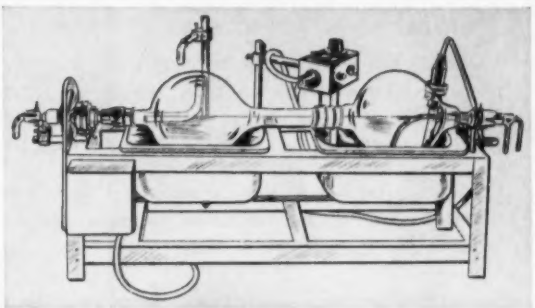

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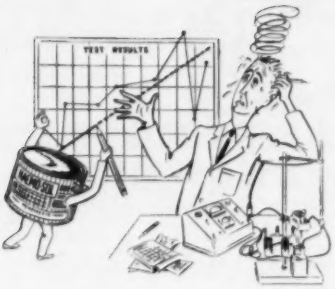

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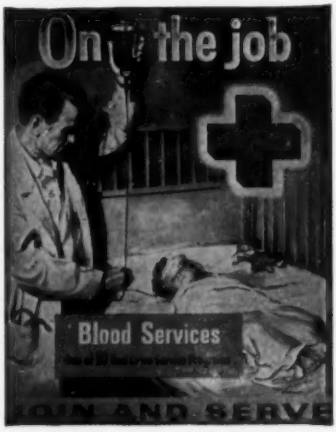

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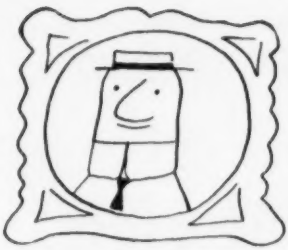

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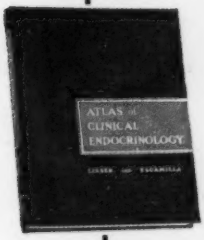

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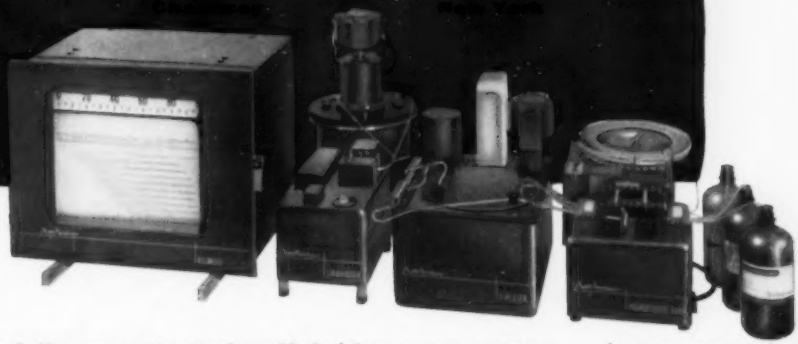

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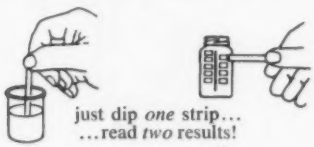

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